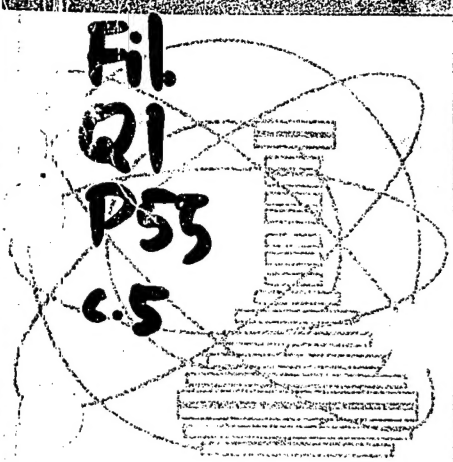

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The cover, taken from the fifth manuscript (Figure 3) published in this issue, is a young spore with sloughed off wall layer (arrow). Note the roughness of the evanescent layer. Scale=26μm

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Antimutagen and Antifungal Compounds from Cosmos caudatus

CONSOLACION Y. RAGASA, ZENAIDA D. NACPIL
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ABSTRACT

The chloroform extract of the leaves of Cosmos caudatus afforded costunolide, stigmasterol, lutein and 4,4'-bipyridine. The structure of costunolide was elucidated by NMR spectroscopy and mass spectrometry, while its relative stereochemistry was deduced from NOESY. The structures of stigmasterol, lutein and 4,4'-bipyridine were identified by comparison of their ^{1}H NMR spectral data with those of the known compounds.

Costunolide was tested for antimutagenicity potential by the Micronucleus test. Results of the study indicated that at a dosage of 8.0 mg/kg mouse, costunolide reduced the number of micronucleated polychromatic erythrocytes induced by mitomycin C by 85%. Previous studies indicated a 79% reduction in MPCE for stigmasterol, while lutein showed an 81% reduction in MPCE. On the other hand, 4,4'-bipyridine did not exhibit antimutagenic activity.

Antimicrobial tests on costunolide indicated a complete inhibitory activity against Staphylococcus aureus and Saccharomyces cerevisiae, partial inhibitory activity against Bacillus subtilis, slight inhibitory activity against Candida albicans and negative inhibitory activity against Escherichia coli and Pseudomonas aeruginosa at concentrations of 100 µg/mL and 1 mg/mL. On the other hand, stigmasterol and 4,4'-bipyridine indicated slight inhibitory activity against C. albicans and S. cerevisiae and negative inhibitory activity against S. aureus, E. coli, B. subtilis and *P. aeruginosa. Lutein was tested for antimicrobial activity in an earlier study and showed high antimicrobial activity.*

KEYWORDS: *Cosmos caudatus*, costunolide, stigmasterol, lutein, 4,4'-bipyridine, antimicrobial, antimutagen, Micronuleus test

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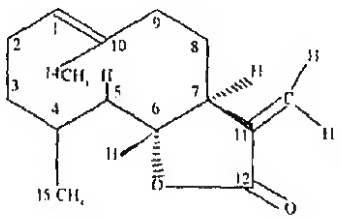
INTRODUCTION

Cosmos caudatus is a common weed throughout the Philippines. It was introduced to the Philippines from Central America. The leaves of the plant are reported to have antimicrobial properties [1]. Earlier studies reported the isolation of amino acids and proteins from *Cosmos caudatus* [2]. *C. hybridus* and *C. sulphureus* afforded costunolide [3]. *C. sulphureus* was reported to contain a range of phenolic compounds, amino acids, fatty acids, and glycosides [4]. *C. bipinnatus* was reported to contain flavones [5] and amino acids [6]. We now report the isolation of costunolide (1), stigmasterol (2), lutein (3) and 4,4'-bipyridine (4) from the chloroform extract of the leaves of the plant.



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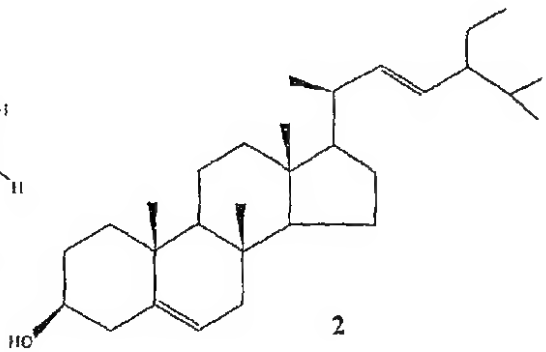

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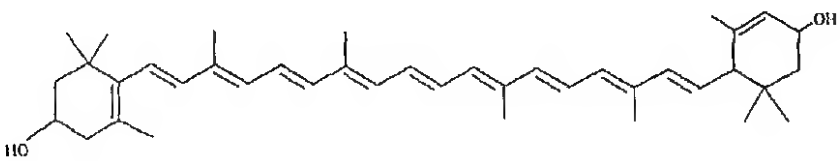

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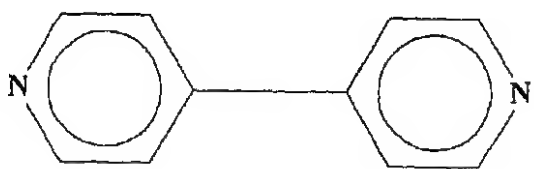

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RESULTS AND DISCUSSION

Cosmos caudatus afforded costunolide (1), stigmasterol (2), lutein (3) and 4,4'-bipyridine (4). The structure of 1 was elucidated by NMR (^{1}H, ^{13}C, COSY) and UV spectroscopy and mass spectroscopy. The relative stereochemistry of 1 was deduced from NOESY.

The ^{13}C NMR spectrum of 1 indicated the presence of fifteen carbon atoms with the following functionalities: a carbonyl carbon of a lactone (δ

171.0), three non-protonated (δ 141.5, 140.0 and 137.0) and three protonated (δ 127.4, 127.1 and 120.0) olefinic carbons, a carbon singly bonded to oxygen (δ 82.0) and seven aliphatic carbons (two of which were methyl carbons which can be verified from the ^{1}H NMR spectrum).

The ^{1}H NMR spectrum of **1** revealed two methyl singlets (δ 1.40, 1.69), four olefinic protons [δ 4.85 (dd, 11.8, 4.7 Hz), 4.74 (δ, 10.6 Hz), 5.51 (δ, 3.5 Hz), 6.25 (δ, 3.5 Hz)] and a lactonic hydrogen [δ 4.58 (dd, 10.6, 9.4 Hz)]. The COSY spectrum indicated two fragments of the molecule as follows: The allylic methyl group at δ 1.40 (3H, s, H-14) was coupled to the olefinic proton at δ 4.85 (H-1), which was coupled to the methylene protons at δ 2.29 (1H, m, H-2a) and 2.01 (1H, m, H-2b), which were in turn coupled to the methylene protons at δ 2.41 (1H, dd, H-3a) and 2.17 (1H, m, H-3b). The coupling chain was blocked by non protonated carbons in both directions. The second fragment of the molecule started with the olefinic protons at δ 6.25 (1H, d, H-13a) and 5.51 (1H, d, H-13b) which were coupled to the methine proton at δ 2.55 (1H, m, H-7), which was in turn coupled to the lactonic hydrogen at δ 4.58 (1H, dd, H-6) and methylene protons at δ 2.11 (1H, m, H-8a) and 1.65 (1H, m, H-8b). The latter protons were coupled to the methylene protons at δ 2.25 (1H, dd, H-9a) and 2.35 (1H, dd, H-9b), while the lactonic hydrogen at δ 4.58 (1H, dd, H-6) was coupled to the olefinic hydrogen at δ 4.74 (1H, d, H-5).

Joining together the two fragments of the molecule to conform with the isoprene rule resulted in structure **1**. Extensive literature search indicated that **1** is costunolide as shown by similar ^{1}H NMR spectra [7].

The relative stereochemistry of **1** was determined by NOESY which indicated correlation through space of the ^{1}H nuclei in the molecule (Fig. 1).

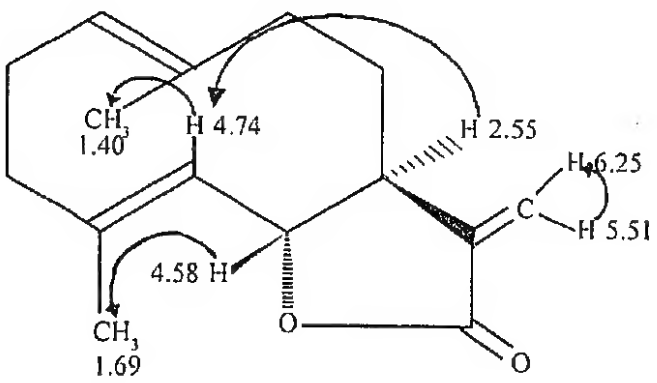

Figure 1. Correlation of ^{1}H NMR Nuclei of 1 from NOESY.

The ^{1}H NMR spectral data of **2** showed resonances at δ 5.11 (1H, dd), 5.17 (dd) and 5.34 (s, br) which were assigned to olefinic protons. The multiplet at δ 3.52 indicated a proton attached to a hydroxyl bearing carbon. Six methyl singlets were found at δ 1.02 (3H), 0.70 (3H), 1.00 (3H), 0.85 (6H) and 0.83 (3H). These are the typical resonances of a sterol. The structure of **2** was deduced by comparing its ^{1}H NMR spectral data with those found in the literature for stigmasterol [8]. The spectra matched in all essential respects, thus **2** is stigmasterol.

Compound **3** indicated λ_{max} at 474 and 445 nm which are characteristics of conjugated double bonds typical of carotenoids [9]. The ^{1}H NMR spectrum indicated resonances for conjugated olefinic protons at δ 6.11 to 6.64 and carbinyl protons at δ 4.0 and 4.1. It showed resonances for ten methyl singlets at δ 0.84, 0.97, 1.06, 1.08, 1.24, 1.61, 1.72, 1.90, 1.95 and 1.96. The ^{13}C NMR spectrum showed forty carbon atoms, four of which were overlapping resonances as indicated by strong resonances for two methyl groups and six olefinic carbons. The structure of **3** was elucidated by comparison of its ^{1}H and ^{13}C NMR spectral data with those of lutein previously reported in *Mimosa invisa* [10].

The ^{1}H NMR spectrum of **4** indicated resonances for aromatic protons at δ 7.69 (d, 14.6 Hz) and δ 8.11 (d, 14.6 Hz), while its ^{13}C NMR spectrum showed resonances for protonated olefinic carbons at δ 132.7 and δ 125.0 and nonprotonated olefinic carbon at δ 130.0. Literature search indicated that **4** is 4,4'-bipyridine as evidenced by similar ^{1}H NMR spectral data [11].

The extract of *Cosmos caudatus* was reported to have antimicrobial activity [1]. In view of this, the antimicrobial activity of **1**, **2** and **4** were tested using the disc diffusion method. Compound **3** showed high antimicrobial activity in a previous study [10].

Antimicrobial test on **1** (Table 1) indicated a complete inhibitory activity against a gram positive bacterium, *Staphylococcus aureus* and a yeast-like fungi, *Saccharomyces cereviseae;* partial inhibitory activity against a gram positive bacterium, *Bacillus subtilis*; slight inhibitory activity against a filamentous fungus, *Candida albicans;* and negative inhibitory activity against gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* at concentrations of 100 μg/mL and 1 mg/mL. This implies that **1** can be used to prevent the proliferation of the tissues of any infections caused by these microorganisms. On the other hand, **2** and **4** indicated slight inhibitory activity against *C. albicans* and *S. cereviseae* and negative inhibitory activity against *S. aureus, E. coli, B. subtilis and P.* aeruginosa at the same concentration. All sample free discs which served as solvent control gave negative activities against the six microorganisms. The solvent used for **1** was hexane, while the solvent used for **2** and **4** was 95% ethanol.

Table 1. Antimicrobial test results of 1, 2 and 4.

Sample	*S. aureus*	*P. aeruginosa*	*E. coli*	*B. subtilis*	*C. albicans*	*S. cerevisiae*
1	(+++) 10.5	(-)	(-)	(++) 12	(+) 10	(+++) 10.5
2	(-)	(-)	(-)	(-)	(+) 10	(+) 10.5
4	(-)	(-)	(-)	(-)	(+) 10.5	(+) 10.5
Control	(-)	(-)	(-)	(-)	(-)	(-)

Disc: 10 mm Diameter
Control: sample free disc
(+++) complete; (++) partial; (+) slight inhibitory activity
and (-) negative activity

Sesquiterpene lactones with α-methylene-γ-lactone moiety are known to possess antitumor properties. Since there is a strong correlation between antitumor and antimutagenic properties and **1** possess an α-methylene-γ-lactone moiety, it maybe a potential antitumor compound. Thus, the antimutagenicity potential of **1** was evaluated using the Micronucleus test. Results of the study (Table 2) indicated that **1** reduced the number of micronucleated polychromatic erythocytes by 85% when it was administered at a dosage of 8.0 mg/kg mouse with a known mutagen, mitomycin C to mice of the Swiss strain. Thus, **1** is an antimutagen. The antimutagenicity potential of **2**, **3** and **4** have not been tested because previous studies reported that **2** [12] and **3** [10] showed high antimutagenic activity, while **4** did not indicate any antimutagenic activity [13].

Table 2. Effects of 1 on the formation of micronucleated polychromatic erythrocytes induced by mitomycin C.

Sample	Dosage (mg/kg	No. of MPCE/1000 PCE ± Ö*	% Reduction in MPCE
1	8.0	1.22 ± 1.12	85
Mitomycin C DMSO	2.75 7.5 mL/kg	8.10 ± 2.18	

*Determined from 15 slides

EXPERIMENTAL

General:

The identity of **1** was established by spectroscopic methods. Spectra were recorded with the use of Bruker AMX 300 and Hitachi UV-Vis spectrophotometer. All NMR spectra were recorded in $CDCl_3$, ^1H NMR at 300 MHz and ^{13}C NMR at 75 MHz. Fractions were monitored by TLC

and spots were visualized by spraying the TLC plates with vanillin/H_2SO_4, then warming. The melting point was obtained by the use of Fisher-Johns melting point apparatus. The optical activity was obtained by the use of Optical Activity Ltd. Automatic Polarimeter.

Sample Collection and Extraction

Cosmos caudatus was collected at Angeles City, Pampanga in June 1992. Three hundred grams of ground dried leaves were soaked in $CHCl_3$. The extract was filtered and the filtrate was concentrated *in vacuo* to afford a crude extract (32 g).

Isolation

The crude extract (10g) was fractionated by vacuum liquid chromatography (VLC) with the use of increasing proportions of ethyl acetate in petroleum ether (10% increment) as eluent. The 100% petroleum ether fraction was rechromatographed in the same solvent to afford 4 (52.5 mg). The 10% and 20% ethyl acetate in petroleum ether fractions were rechromatographed using solvent systems containing 5%-20% ethyl acetate in petroleum ether to afford 2 (71.7 mg). The more polar fraction was rechromatographed using 15% ethyl acetate in petroleum ether to afford 1 (341 mg). The fractions obtained using 30 % and 40 % ethyl acetate in petroleum ether fractions were rechromatographed in 35% ethyl acetate in petroleum ether to afford 3 (21.3 mg) after recrystallization from diethyl ether.

Bioassay

A. Antimicrobial Test

Test bacteria in nutrient broth were cultured and incubated at 37°C overnight. Culture broth bacteria were incorporated in molten nutrient agar 40° to 42°C at 2% concentrations of the test organisms to medium. Seeded media were transferred in 10-ml portions to properly labeled petri dishes. Test plates were preincubated for 1 hour at 37°C before conducting tests. Three separate discs were dipped into the test samples at a concentration of 100 µg/ml and placed in proper distances upon the surface of the agar to allow development of inhibition zones. Compound 1 was also tested at a concentration of 1 mg/mL. A fourth disc was dipped in the control (95% ethanol, solvent) and placed upon the agar. All test plates were incubated upside down at 37°C for twenty-four hours. The mean average diameter zones of inhibition produced by each test sample in three replication were measured in terms of milliliters.

B. Micronucleus Test

Mitomycin C (2.75 mg/kg mouse) and isolate 3 (0.200 mg/kg mouse) dissolved in dimethyl sulfoxide (DMSO) (7.5 mL/kg mouse) were administered orally to mice of the Swiss strain (7-12 weeks) from DOST. For the control, only the mutagen, Mitomycin C (positive control) and

DMSO (solvent control) were administered orally to the same strain of mice. For each isolate and control, five mice were tested. The second administration was carried out after twenty-four hours. Six hours after the second administration, the mice were sacrificed and blood from the bone marrow was flushed with fetal calf serum.. Blood from the bone marrow was smeared on slides, three slides per mouse were prepared. The slides were stained with undiluted May-Gruenwald solution, followed by 50% May Gruenwald solution, then 15% Giemsa stain [14]. The number of micronucleated polychromatic erythrocytes (MPCE) per 1000 polychromatic erythrocytes (PCE) were then counted with the use of a high power microscope, and results are given as % reduction in MPCE.

CONCLUSIONS

The chloroform extract of the air-dried leaves of *Cosmos caudatus* afforded **1-4**. Micronucleus test on **1** indicated that it has high antimutagenic activity. Compounds **1**, **2** and **4** were tested for antimicrobial activity against five microorganisms. Compound **1** showed activity against *S. cereviseae, S. aureus, B. subtilis* and *C. albicans*, but was found inactive against *E. coli* and *P. aeruginosa.* Compounds **2** and **4** showed activity against *C. albicans* and *S. cereviseae*, but was found inactive against the other four microorganisms.

ACKNOWLEDGMENT

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Anticarcinogenicity and Antiteratogenicity Potential of the Antimutagenic Chloroform Leaf Extract from Mentha cordifolia Opiz.

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ABSTRACT

The chloroform leaf extract of Mentha cordifolia leaves (YBFC) was tested for its antiteratogenic and anticarcinogenic potentials. The antiteratogenic and antitumorigenic activities were monitored using the dominant lethal test and mouse skin cancer assay, respectively. At 2.5 mg/20 g mouse, YBFC increased to 100% the frequency of fertile matings, decreased to 0% the number of females with resorptions (early deaths), and decreased to 3.4% the number of dead implantations. At 0.05 mg / 0.2 ml acetone, YBFC decreased the incidence of skin tumors by 44.4% when applied immediately after croton oil. It afforded additional protection when applied daily for five days prior to the use of dimethylbenzanthracene (DMBA) as there was a 58.3% reduction in tumor incidence. Hence, YBFC showed antiteratogenic and anticarcinogenic potentials.

INTRODUCTION

Teratogenicity is due to the chromosomal damage of the germ cells. Its manifestations are embryolethality, birth defects, growth retardation, functional alterations of the nervous, immune, and endocrine systems, and childhood cancer.

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Many cancers are caused by a two stage process through exposure to substances known as initiators and promoters. Genotoxic effects on somatic cells has been associated with the initiation of cancer. Research scientists are exploring ways of interrupting these processes to prevent the development of cancer.

Mentha cordifolia Opiz., commonly known as peppermint, marshmint, or yerba buena, is an aromatic herb of the Lamiaceae family. Its leaves may have the potential to counteract the effects of teratogens and carcinogens. At 2 mg / 20 g mouse, the chloroform extract of yerba buena leaves is antigenotoxic as it reduced the number of micronucleated polychromatic erythrocytes induced by tetracycline, a known mutagen, by 61.1% (Villaseñor et al., 1995). The importance of this study lies in the counteraction of the mutagenic activity of environmental chemicals and in

the probable anticarcinogenic and antiteratogenic activities of antimutagens. An extensive search of the literature showed that the structure of these bioactive constituents are not yet elucidated.

MATERIALS AND METHODS

Plant Extracts

The yerba buena leaves were purchased from the National Research Council of the Philippines (NRCP) in Bicutan, Taguig, Metro Manila. Approximately 1 kg of leaves were air-dried and then homogenized in methanol (10 l). The methanol extract was filtered and then concentrated in vacuo at 40°C using a rotary evaporator (Heidolph). The methanolic fraction was partitioned between hexane and water (6:1). The aqueous layer was further extracted with chloroform (6X) and then with ethyl acetate (6X). The hexane, chloroform, and ethyl acetate portions were then concentrated in vacuo to give fractions FB, FC, and FD, respectively.

The required weight of the chloroform extract were dissolved in dimethysulfoxide (DMSO) for the antiteratogenicity and in acetone for the antitumorigenicity study.

Bioassay for Antiteratogenicity Potential: Dominant Lethal Test (Searle, 1974)

Swiss Webster mature males aged 6-7 weeks and virgin female albino mice were used as test animals. The males were treated only with tetracycline, a known teratogen (Sylianco and Blanco, 1984), using 55 mg / kg mouse. They were then injected intraperitoneally with the chloroform extract from yerba buena leaves using 2.5 mg / 20 g mouse. The treated males were mated to two females on the 6th day after treatment. Five males were used per treatment.

After 21 days, the pregnant females were sacrificed by cervical dislocation. The abdomen was opened and the uterine horns were located. The number of live and dead implants were recorded, the dead being classified as early or late death. The fetuses were then removed from their fetal membranes and placed in bottles with Bouin's fixative for at least 1 week. The preserved fetuses were subjected to gross analysis: 2 eye slits, 2 ears, lower jaw slightly shorter than the snout, 4 legs which point in the proper direction, 4 digits on each front paw, 5 digits on the hind paw, an anal and genital opening, a tail, an umbilicus, and a smooth epidermis with no protrusions.

Bioassay for Anticarcinogenicity Potential: Skin Cancers (Mehta et al., 1995)

Albino mice were used as test animals. The back of the mouse was shaved. After 6 days, dimethylbenzanthracene (DMBA) (Sigma) (410 µg/0.2 ml acetone), a known initiator, was applied on the shaved portion. On the fourth day, croton oil (Sigma)(0.03% in acetone), a known promoter, was

applied 3 times a week for 20 weeks. The chloroform extract (0.05 mg/ 0.2 ml acetone) was splashed using three different protocols: 5 days prior to DMBA application, one hour prior to croton oil, and immediately after croton oil. Ten mice per use per treatment and per test protocol.

RESULTS AND DISCUSSION

There are four criteria for teratogenicity using the dominant lethal test: 1) an increase in the frequency of dead implantations; 2) reduction in the average number of living embryos; 3) reduction in the average number of implantations; and 4) reduction in the frequency of fertile matings (Generoso, 1976). The scoring of dominant lethals may either be direct or indirect. The direct scoring must show an increased incidence of early fetal deaths while in the indirect scoring there is an increased incidence of pre-implantation losses. Antiteratogens should counteract the aforementioned four criteria of teratogenicity.

The results of the dominant lethal test are summarized in Table 1. At 2.5 mg/ 25 g mouse, the chloroform leaf extract increased the fertility and gestation indices to 100% and 96.6%, respectively. These suggest that there were more pregnant females and more live implants in a litter compared to the solvent control (tetracycline + DMSO) group. Moreover, lethality index (3.4%) was relatively low indicating a higher ratio of pregnant females and

Table 1. Antiteratogenicity potential of the chloroform extract of *Mentha cordifolia* Opiz. leaves.

	tetracycline (55 mg / kg)	tetracycline + DMSO	tetracycline + chloroform extract (2.5 mg / 20 g)
Fertility Index	60	60	100
Implantation Index	9.67	11.67	9.83
Gestation Index	77.6	90.0	96.6
% Dead Implants	22.4	10.0	3.4
% Female with Resorptions	83.3	50.0	0.0
Average Fetal Weight (g)	0.36	0.66	0.74

Fertility Index = no. of females pregnant / no. of females mated x 100
Implantation Index = total implantations / no. of pregnant females
Gestation Index = no. of live implantations / total no. of implantations x 100
% Dead Implants = 100 - Gestation Index
% Female with Resorptions = no. of females with resorptions / no. of females pregnant x 100
(Note: resorptions refer to early deaths)

live implants compared to the solvent control group (10.0%).

These observations imply that the chloroform extract was able to counteract the teratogenic effect of tetracycline as there was an increase in the frequency of fertile matings from 60% to 100%, a decrease in the number of females with resorptions (early deaths) from 50% to 0%, and a decrease in the number of early and late deaths from 10% to 3.4%.

In the mouse skin cancer assay, dimethylbenzanthracene (DMBA) was used as the initiator which induces irreversible genetic alterations. Croton oil, the crude source of tetradecanoylphorbol acetate (TPA), was used as the promoter and was repeatedly applied for twenty weeks. These resulted in clonal expansion of initiated cells in the form of tumors.

Table 2 summarizes the results of the mouse skin cancer assay. The tumors were counted once a week and their sizes measured on the twentieth week. There was a 90% tumor incidence for the positive control group (DMBA + croton oil + acetone). The % tumor incidence decreased by 20.7 % upon application of 0.05 mg of YBFC one hour prior to application of croton oil.

Table 2. Antiteratogenicity potential of the chloroform extract of *Mentha cordifolia* Opiz. leaves.

Test Sample	Test Protocol	% Tumor Incidence*	Ave. No. of Tumors per Mouse	Ave. Diameter of Skin Tumors (mm)	Mortality (%)
Spontaneous control		0.0			10.0
DMBA + croton oil + acetone		90.0	2.2	1.9	0.0
DMBA + croton oil + YBFC	YBFC applied daily for 5 days prior to DMBA and one hour prior to croton oil	37.5	2.0	1.8	20.0
DMBA + croton oil + YBFC	YBFC applied for one hour prior to croton oil	71.4	1.4	2.2	30.0
DMBA + croton oil + YBFC	YBFC applied immediately after croton oil	50.0	1.3	4.0	40.0

YBFC - $CHCl_3$ extract of yerba buena leaves

*% tumor incidence = no. of mice with tumors / no. of surviving mice

There was a 44.4% decrease in % tumor incidence when the same concentration of YBFC was applied immediately after croton oil. Application of the same concentration of YBFC daily for five days prior to DMBA reduced the % tumor incidence by 58.3%. These data implies that YBFC is not a co-carcinogen nor a co-tumor promoter as there was no increase in the incidence of tumors after YBFC application. Moreover, additional protection is provided when YBFC was applied prior to DMBA as there was a decrease in tumor incidence from 50% to 37.5%. No histopathological evaluations were made on the skin tumors.

Figure 1 shows the time tumor occurrence while Figure 2 shows the number of tumors in individual mouse. For the positive control, two tumors appeared on the eleventh week and peaked on the fourteenth week where four mice developed a total of 5 tumors. For the YBFC treated groups, most of the tumors first appeared on the fourteenth week. Hence, in general, there was a

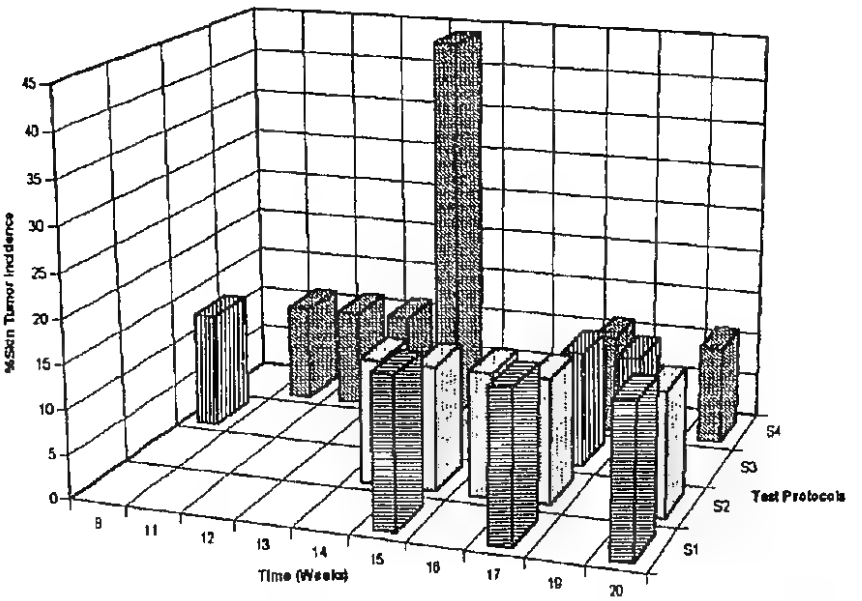

Figure 1. % skin tumor incidence vs time in weeks.

S1 = YBFC applied immediately after croton oil
S2 = YBFC applied one hour prior to croton oil
S3 = YBFC applied daily prior to DMBA; one hour after croton oil
S4 = positive control (DMBA + croton oil + acetone)

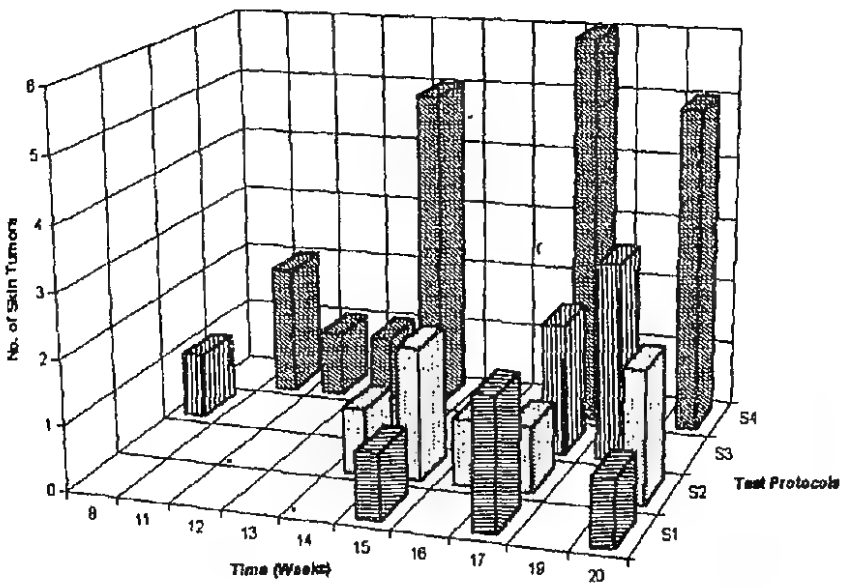

Figure 2. Number of skin tumor per mouse vs time in weeks.

S1 = YBFC applied immediately after croton oil
S2 = YBFC applied one hour prior to croton oil
S3 = YBFC applied daily prior to DMBA; one hour after croton oil
S4 = positive control (DMBA + croton oil + acetone)

delay and a reduction in the formation of tumors for the groups treated with the chloroform extract of yerba buena leaves.

CONCLUSION

The antimutagenic chloroform leaf extract of *Mentha cordifolia Opiz.* (YBFC) at a dosage of 2.5 mg / 20 g mouse, showed an antiteratogenic potential as it increased the frequency of fertile matings, decreased the number of females with resorptions, and decreased the number of dead implantations. It is not a co-carcinogenic nor a co-tumor promoter as there was no increase in the incidence of skin tumors after YBFC application. It decreased the incidence of skin tumors by 44.4% when applied immediately after croton oil. If afforded additional protection when applied daily for five days prior to DMBA as there was a 58.3% reduction in tumor incidence. In this preliminary study, we have evaluated the anticarcinogenicity and antiteratogenicity potential of the chloroform leaf extracts of *M. cordifolia.* Our long range study includes the purification and structure elucidation of these bioactive constituents.

ACKNOWLEDGMENT

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Oxidative Dehydrogenation of Ethanol Over Mixed Metal Oxides

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ABSTRACT

Oxidative dehydrogenation of ethanol to acetaldehyde and acetic acid is studied in heterogenous catalytic system over the catalysts containing varying proportion of antimony oxide in the mixture of tin and molybdenum oxides considering lower explosive limit. About 20% mole of Sb_2O_4 is shown to have excellent activity for partial oxidation of ethanol to acetaldehyde and acetic acid at 280°C. There is complete ethanol conversion up to 20 mole% Sb_2O_4, increase in W/F increases selectivity towards acetic acid. Further increase in the proportion of Sb_2O_4 shows increase in selectivity towards acetaldehyde whereas there is no substantial change towards the selectivity of acetic acid.

INTRODUCTION

In recent years energy conservation in process plants has attracted the attention of scientists and engineers throughout the world due to the sharp increase in the cost of energy. Therefore new and improved catalysts have to be developed to minimize process steps and to use milder reaction conditions in the chemical industry. The present work is an attempt to develop an improved catalyst for the oxidative dehydrogenation of ethanol to acetaldehyde and acetic acid. Acetaldehyde is an important industrial intermediate used for the production of large number of chemicals such as chloral, acetic acid and acetic anhydride etc. Acetic acid is an important organic chemical finding its way into a vast range of end products. The most important derivative vinyl acetate is used in variety of polymers which have become major components of paints, adhesives and textiles. Acetic acid itself is used as a solvent in the production of terephthalic acid and is used widely in food and pharmaceutical applications.

We have studied and presented here the work on tin-molybdenum-antimony mixed oxide catalysts for the oxidative dehydrogenation of ethanol using a heterogenous system.

MATERIALS AND METHODS

Starting Materials

The starting materials for the catalyst preparation were tin (II) chloride $SnCl_2.2H_2O$ (LR), ammoniuim molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ (AR) and antimony (III) oxide Sb_2O_3 (all from Merck) and were used as received.

Air (cp) and absolute alcohol (AR) were used for the measurement of catalytic activity.

Preparation of Oxides

(i) Pure α-Sb_2O_4 (BET surface area $2M^2/g$ where BET stands for Brunauer Emett Teller) was obtained by calcination of SB_2O_3 in air at 500°C for 20h; (ii) SnO_2 was prepared by precipitation of an aqueous solution of $SnCl_2.2H_2O$ with ammonia at pH ~ 7.5 followed by drying at 110°C for 16h and calcining at 600°C for 8h; (iii) MoO_3 was obtained by thermal decomposition of $(NH_4)_6\ Mo_7O_{24}.4H_2O$ in air at 500°C for 20h. Mechanical mixtures were obtained by vigorously mixing the suspension of the powders in n-pentane for 10 minutes by means of a mixer (Ultra-Turmax). After evaporation of the solvent under reduced pressure the mixture obtained were finally dried at 80°C overnight. No further calcination was carried out. The oxide mixture was pressed into the form of a pellet and subsequently cut into suitable 10-20 mesh size for the measurement of catalytic activity. Table 1 shows the chemical composition of the catalysts.

Table 1. Chemical composition of the catalysts.

Catalyst	Composition (more proportion) SnO_2: MoO_3: Sb_2O_4	Sb_2O_4 (mole %)	Surface area M^2/g	
			Before Test	After Test
Catalyst I	6.7 : 4.4 : 1.1	9.0	2.86	2.75
Catalyst II	6.7 : 4.4 : 2.7	19.6	-	-
Catalyst III	6.7 : 4.4 : 5.3	32.3	-	-

Characterisation

The X-ray diffraction analyses of the catalysts were carried out on Rigaku D Max/III VC diffractometer using Ni filtered CuK α (λ = 1.5404 Å radiation). The BET surface areas of the catalyst I were measured at -196°C using N_2 after evacuating the catalyst at 300°C for 1h. They were depicted in Table 1.

Catalytic Activity Measurements

The catalytic reaction was carried out at atmospheric pressure with a downflow integral silica reactor which was heated with a metal block furnace of 0.3 m constant temperature zone. Air was passed over the catalyst through a ethanol saturator maintaining at 18°C. Temperatures were recorded by means of a precalibrated chromel-alumel thermocouple.

The reactor was charged with catalyst (23 g of 10-20 mesh size) along with porcelin beads and heated in a flow of dry air. The reactor

temperature was slowly increased up to the desired level (280°C) and ethanol was introduced by passing air through the saturator. The liquid product was condensed through the ice cold trap. The exit gas was absorbed for known time in the known quantity of saturated sodium metabisulphite solution.

The liquid and gas analyses were carried out with a gas chromatograph (Shimadzu GC R15A) equipped with porapak N column and TCD. The acetic acid in liquid product was estimated volumetrically using standard sodium hydroxide solution. The acetaldehyde was estimated by absorbing in excess sodium metabisulphite solution. Excess metabisulphite was back titrated with standard iodide solution. Acetaldehyde was calculated by knowing the consumed metabisulphite quantitatively.

RESULTS AND DISCUSSIONS

Characterization

The value of 'd' spacing and the relative intensities of peaks with respect to the most intense peak at 2θ = 26.6 for SnO_2, 2θ = 25.7 for MoO_3 and 2θ = 29.1 for α-Sb_2O_4 are shown in Table 2, 3 and 4 and in agreement with literature (XRD data Amer. Soc. 1955). The XRD data for SnO_2-MoO_3-αSb_2O_4 system (before test) is shown in Table 5. There is no bulk reaction or solid state reaction between α-Sb_2O_4, SnO_2 and MoO_3. There is neither formation of a new phase nor a surface contamination is observed. Also there is no formation of $SnSbO_4$ and α-Sb_2O_4 remains unchanged. The tested mixtures consist of α-Sb_2O_4, SnO_2 and MoO_3. The particles are simply in physical contact.

Table 2. X-ray diffraction data for SnO_2.

2θ	d	Peak Height	I/I_0(%)
26.6	3.34	29	100
33.9	2.64	22	75.9
51.8	1.76	16	55.2

Table 3. X-ray diffraction data for MoO_3.

2θ	d	Peak Height	I/I_0(%)
12.8	6.9	29	64.4
25.7	3.46	45	100
27.4	3.25	41	91.1
39.0	2.30	22	48.9

Table 4. X-ray diffraction data for α–Sb_2O_4.

2θ	d	Peak Height	I/I_0(%)
25.9	3.43	10.5	33.3
29.1	3.06	31.5	100
30.4	2.93	11.0	34.9
33.8	2.64	5	15.9
48.9	1.86	5	15.9
51.3	1.78	5	15.9
53.2	1.72	6.5	20.6

Table 5. X-ray diffraction data for SnO_2-$MoO_3\alpha Sb_2O_4$.

2θ	d	Peak Height	I/I_0(%)
25.6	3.4	4.5	40.91
26.5	3.33	9.5	86.36
27.2	3.27	7.5	68.18
29.0	3.06	4.0	36.36
30.2	2.93	1.5	13.64
33.8	2.65	11.0	100

Catalyctic Activity Measurement

The product of partial oxidation of ethanol mainly consisted of CO_2, acetaldehyde and acetic acid. Table 6 shows that with catalyst I and II the selectivity towards acetic acid is decreased upon decreasing W/F. However, with catalyst III there was no substantial change. The reduction in selectivity of acetic acid is as expected due to less contact time concurrently producing acetaldehyde, the precursor for acetic acid formation. In the case of catalyst III the constant selectivity for acetic acid may be due to the higher concentration of antimony oxide (α-Sb_2O_4) which is discussed in the subsequent section. The increase in exothermicity (Δ T°C) at higher space velocity (lower W\F) is as expected. However, it is observed from the results that the surface area of the catalyst I is slightly

Table 6. Activity performance of the catalysts.

	Catalyst-I			Catalyst-II			Catalyst-III		
W/F gh mole $^{-1}$	40	55	80	40	55	80	40	55	80
Δ T (°C)	50	40	20	50	40	30	60	50	40
%S CH_3CHO	7.3	16.2	10.7	30.9	33.3	6.2	25.3	32.7	40.6
%S CH_3COOH	36.4	39.4	44.8	26.0	35.3	36.1	23.2	25.5	26.6

reduced after the catalytic test. This reduction in surface area is attributed to the sintering of the catalyst during testing.

Effect of Antimony Oxide

It has been proposed that in the absence of Sb_2O_4 the oxides are reduced continuously giving rise to deeper reduced structure by way of degradation of crystallites of metal oxides (Weng et al. 1991; Zhou et al. 1991). Sb_2O_4 produces mobil oxygen species which migrate to the surface of other oxides e.g. SnO_2, MoO_3 where these create and/or regenerate the catalytic selective sites (remote control mechanism). Spillover oxygen also protects compound oxides from segragation. It can be stated that Sb_2O_4 acts as a donor while other oxide as an acceptor. A $18O_2$ study showed that migration of oxygen species takes place from Sb_2O_4 to MoO_3/SnO_2 (Weng et al., 1989). This migration helps oxidation of reduced sites. Thus catalyst-III having greater Sb_2O_4 amount (32.3% mole) shows constant selectivity for acetic acid than catalyst I and II which contain 9 and 19.6 mole% of Sb_2O_4 respectively.

CONCLUSIONS

This study shows that low contact time and higher content of antimony oxide (32 mole%) in oxide mixture of two metalic elements (Sn,Mo) exhibit higher selectivity (~ 67%) towards acetaldehyde and acetic acid. Sb_2O_4 brings about increase of activity and selectivity due to spillover oxygen.

The mixture of SnO_2 and MoO_3 containing α-Sb_2O_4 about 32 mole% seems to be a potential catalyst for oxidative dehydrogenation of ethanol.

This process also shows its uniqueness of operation at lower temperature of 280°C for oxidative dehydrogenation of ethanol to acetaldehyde with complete conversion as compared to conventional silver catalyst operating at higher temperature of 300-575°C with lower ethanol conversion per

pass of 25-35% (Lowenheim and Moran, 1975).

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Seaweed Tablet: A Natural Source of Iodine

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ABSTRACT

Species of seaweeds namely: Halymenia durvillaei, Laurencia flexilis and Sargassum gigantifolium were processed into dried form and formulated as tablet. Prior to tablet formulation, the seaweeds were assayed for iodine and trace elements. The seaweeds that exhibited significant values of iodine and trace elements were further analyzed for the presence of heavy metals followed by acute oral toxicity test (LD_{50})

Among the seaweeds evaluated, H. durvillaei was found to contain high level of iodine (0.255 % w/w) and magnesium (1.65% w/w) with sufficient amount of zinc (25.69 ppm) and phosphorus (11.68 ppm). Analysis of heavy metals showed minute amount of mercury (0.0055 ppm), cadmium (0.67 ppm) and lead (1.80 ppm). The median lethal dose (LD_{50}) of H. durvillaei administered orally in Swiss male mice is 119.1489 ± 4.9873 g/kg.

Tablet formulation was based on the U.S. recommended daily allowance of 0.15 mg of iodine per adult and children. The final product was comparable to imported Kelp pills (available in the local market) in terms of physical properties and iodine content.

INTRODUCTION

Iodine Deficiency Disorder (IDD) is one of the major nutrition problems that plague our country according to the studies conducted by the Division of Nutrition of the Department of Health (DOH, 1987). The studies also showed that many Filipinos are suffering from iodine deficiency disorder and iodine plays an important role in the cure of goiter. The goiter rate increased from 1976 to 1981 with a prevalence range of 32.9% to 66.4% in adult women and affects mainly pregnant and lactating women (DOH, 1987). A survey conducted by the Department of Health showed that in 1987 Kalinga Apayao registered a prevalence range of 12.18% of goiter case, Ifugao with 20.38% and Quirino with 17.14%. A study jointly conducted by the

Department of Health and the Department of Education Culture and Sports (DOH, 1987) revealed an average goiter rate of 12.18% which showed that many provinces have a goiter prevalence above 50% with the highest of 93%.

Possible intervention of this disease is through inclusion of iodine in the daily diet, food fortification, iodine supplementation, nutrition promotion and advocacy. It had been observed that people who included seaweeds in their daily diet are not readily affected with this type of disease since these algae contain large amount of iodine. According to Booth (1979), the first commercial source of iodine came from marine plants. These red and brown algae are abundant in our country but unfortunately not all Filipinos are fond of eating these plants in raw form. Also, these plants are seasonal. The transformation of these algae into tablet form which is the main objective of this study could probably be applied to treat or minimize the iodine deficiency disease. Nowadays, people are more interested in using natural products than synthetic ones because of inherent non-toxic effects. Chapman (1980) reported that there has been a claim that iodine is readily absorb by human and animals from seaweeds than from other sources.

The Philippines is richly endowed with red and brown seaweeds. These grow widely in most parts of the country and are mostly edible. As reported by De Leon et.al. (1963), these contain iodine as tabulated in Table 1. The distribution of these red and brown seaweeds in the country is shown in Table 2 as reported by Silva et. al. (1987). Sargassum according to Cordero (1981), is generally seasonal, although some species appear to "persist" the whole year round.

Table 1. Chemical composition of some Philippine algae. (De Leon et. al., 1963).

Specimen	% Crude Fat	% Crude Protein	% Iodine
A. Green Algae			
Codium sp.	1.21	4.97	1.94
B. Brown Algae			
Hydroclathrus clathratus	1.03	8.96	2.09
Sargassum ilicifolium	0.47	7.06	2.85
Turbinaria trialata	1.63	7.17	·
C. Red Algae			
Eucheuma muricatum	1.59	6.75	2.82
Gracilaria salicornia	0.78	10.62	1.90
Halymenia durvillaei			
(Bleached)	0.53	11.87	5.39
(Fresh)	0.66	12.75	6.21
Hypnea musciformia	0.75	10.34	2.49

Table 2. Philippine distribution of red and brown seaweeds. (Silva et. al, 1987).

Places	*Laurencia*	*Sargassum*	*Halymenia*
Luzon			
Batanes	x		x
Pangasinan	x	x	x
Bataan	x		x
Batangas	x	x	x
Or. Mindoro	x		
Ilocos Norte	x	x	
Cagayan		x	
Ilocos Sur		x	x
La Union		x	
Cavite		x	x
Quezon		x	x
Sorsogon			x
Catanduanes			x
Palawan	x		x
Visayas			
Samar	x		x
Leyte	x		
Iloilo	x		
Negros Oriental	x	x	x
Negros Occidental	x	x	x
Masbate		x	
Panay			x
Cebu	x		x
Aklan			x
Guimaras			x
Mindanao			
Surigao	x		
Zamboanga	x	x	x
Surigao del Norte	x		
Sulu	x		x
Tawi-Tawi	x		

Although the seaweeds grow abundantly in the Philippines, no local manufacturer of iodine has been reported. The Food and Nutrition Research Institute of the Department of Science and Technology is currently undertaking studies on iodized water while the Department of Health is implementing its five-year directional plan for iodine deficiency disorders which involves the distribution of iodized capsules or iodine supplements to all endemic areas based on the level of severity. In 1973, the Ministry of Health, NSDB, Philippine Navy and Commission on

Volcanology have agreed to establish a plant for iodized salt in Tiwi, Albay and Bolinao, Pangasinan. However this was discontinued due to lack of financial and political support. Recently, a law was passed by the Executive House to implement the iodization of salt in the whole country.

This study involves the formulation of seaweeds into tablet as source of iodine. Clinical testing was not covered in the study.

MATERIALS AND METHODS

Collection of Samples

Samples of *Halymenia* species were collected in Naic, Cavite (*14 ° 19'* 15.16" Lat and 120 ° 45' 46.09" Long) while *Sargassum* and *Laurencia* species were gathered in Palauig, Zambales (15 ° 26' 10.11" Lat and 119 ° 54' 15.58" Long) based on their Philippine distribution (Silva, et al 1987). Measurements were t aken in a scale of 1:50,000 from the town center to the collection site. Collection was undertaken in the month of *January* and July1993. The samples were then properly identified based on their *herbarium specimen.*

Assay of Iodine Content

Iodine content was determined according to the Official Methods of analysis for Iodine (AOAC, 1990). All solvents and reagents used were of analytical grade.

Assay of Trace Elements

The presence of Ca, Mg, Fe, Zn and P were determined according to the Official Methods of Analysis for Trace elements (AOAC, 1990). All solvents and reagents used were of analytical grade.

Heavy Metals Analysis

Heavy metals such as Hg, Cd, Pb, and As were determined according to the Official Methods of Analysis for Heavy Metals (AOAC, 1990). All solvents and reagents used were of analytical grade.

Acute Oral Toxicity (LD_{50}) Test

Swiss male mice were used in the experiment. A thick red homogenate of *H. durvillaei* was prepared. Preliminary dosing was done to determine the expected dose that will cause death of 50% of the experimental animals. Three (3) increasing log doses of the test substance were administered orally in series to the animals in four (4) groups of ten (10) including the control. The number of deaths and other adverse/abnormal signs and manifestations were closely observed and noted for the first two (2) hours after administration of test sample. This was continued in the next twenty four (24), to forty eight (48) hours daily up to fourteen (14) days. The median lethal dose (LD_{50}) was computed using the Probit Analysis Method by Fisher and Yates, 1952.

Physical Tests for Formulated Tablet

The physical properties of formulated tablets were evaluated according to the Official Methods of the United States Pharmacopeia, XXII (1990). All solvents and reagents used were of analytical grade.

Preparation of Sample

Fresh seaweeds were washed thoroughly with running tap water to remove any adhering dirt and impurities. These were then soaked in 0.2% sodium hypochlorite solution for 15 minutes. The bleaching solution was discarded and the seaweeds were washed again with demineralized water before drying. The dried seaweeds were then ground to powder form.

Formulation Studies

Several formulations were done on the preparation of seaweed tablet (see Table 3). The composition of the seaweed tablet generally consisted of processed seaweeds and calcium stearate, microcrystalline cellulose and stearic acid as the additives. A manual single type pellet press (Parr Instrument Company, USA) was used in forming the tablet.

Table 3. Different formulations prepared for seaweed tablet. (Percent composition)

	I	II	III	IV	V	VI	VII
Seaweeds	45	70	85	67	67	33	37.5
Microcrystalline cellulose	30	28	10	22	13	22	12.5
Stearic Acid	25	2	5	-	11	22	37.5
Calcium stearate	-	-	-	11	9	22	12.5
	100	100	100	100	100	100	100

	VIII	IX	X	XI	XII	XIII
Seaweeds	12	40	33	30	55	70
Microcrystalline cellulose	14	40	55	60	35	24
Stearic Acid	55	13	6	5	5	3
Calcium stearate	19	7	6	5	5	3
	100	100	100	100	100	100

Total Microbial Count

The formulated tablets were analyzed for their microbial content. This was determined according to the Official Methods of United States Pharmacopeia, XXII (1990) for microbial limit test. All solvents and reagents used were of analytical grade.

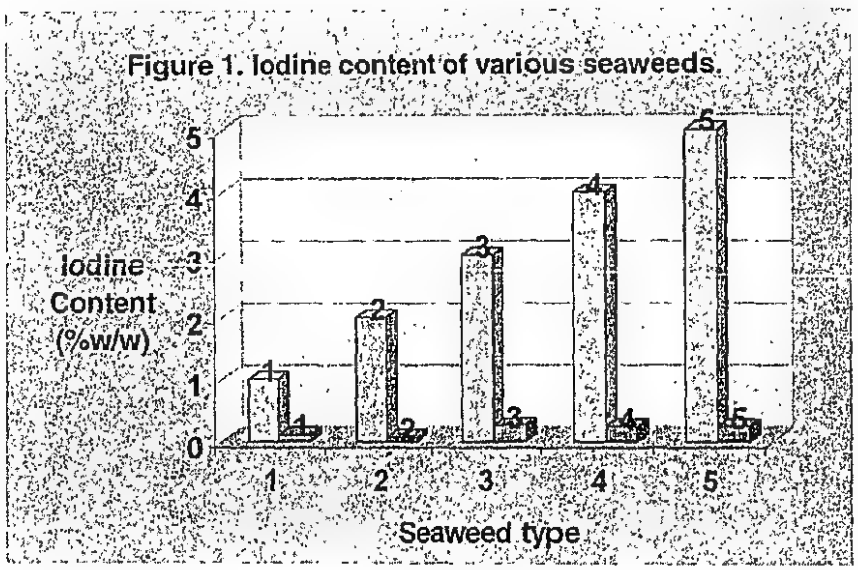
Stability Studies

Seaweed tablets were packed in transparent bottles with cover and without cover. These were then stored in three different conditions namely: room

temperature, 4 ° C and 40 ° C. Iodine content was monitored every two months.

RESULTS AND DISCUSSION

The iodine content of dried seaweed samples are shown in Figure 1. Results demonstrated that *H. durvillaei* gave the highest content of iodine (0.255% w/w) followed by *L. flexilis* (0.232% w/w), *S. gigantifolium* gave the lowest content of iodine (0.058% w/w).



Seaweed samples were also evaluated for their trace elements content (Figs.2-6). Among the seaweeds collected , *L. flexilis* had the highest content of zinc, iron and phosphorus with small amount of magnesium. Calcium is also present in sufficient quantity. *H. durvillaei* contained minimal amount of iron but had higher content of magnesium than *L.*

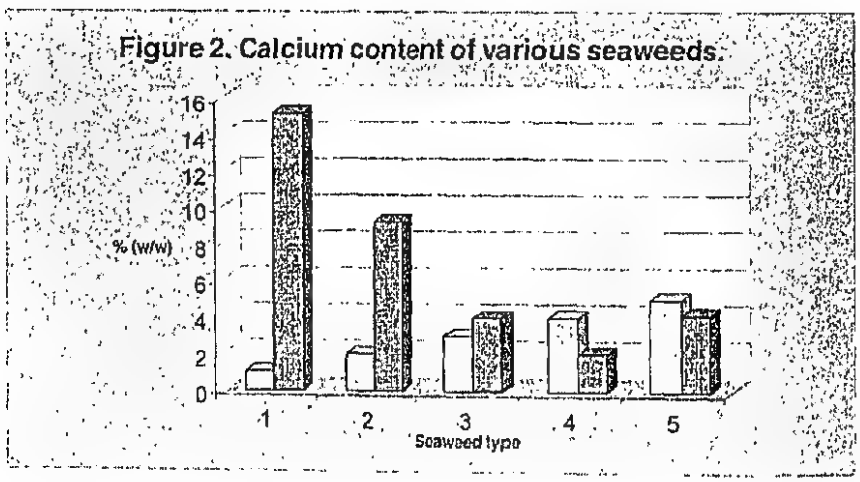


Figure 3. Magnesium content of various seaweeds.

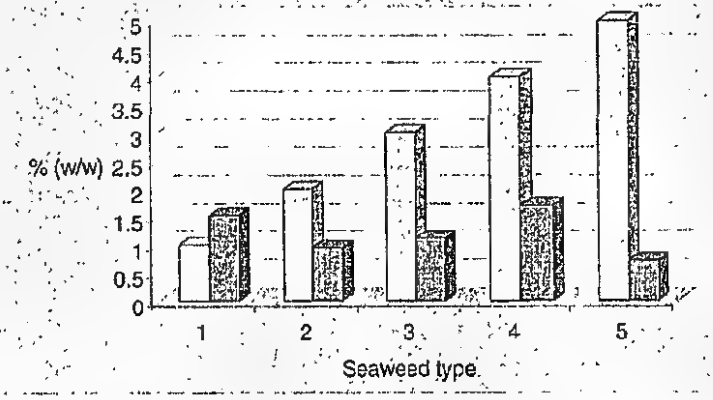


Figure 4. Iron content of various seaweeds.

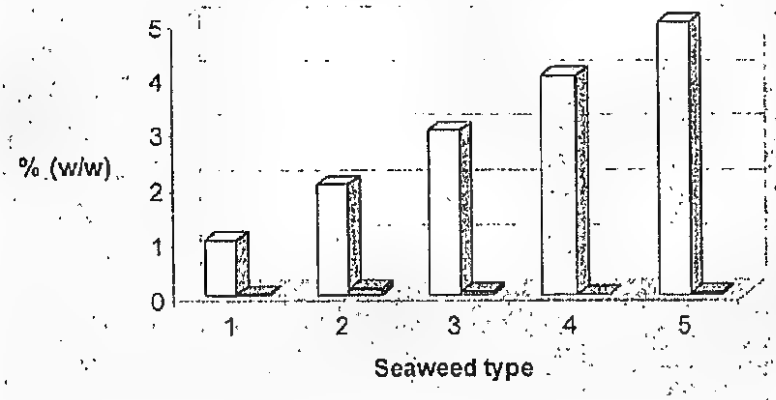
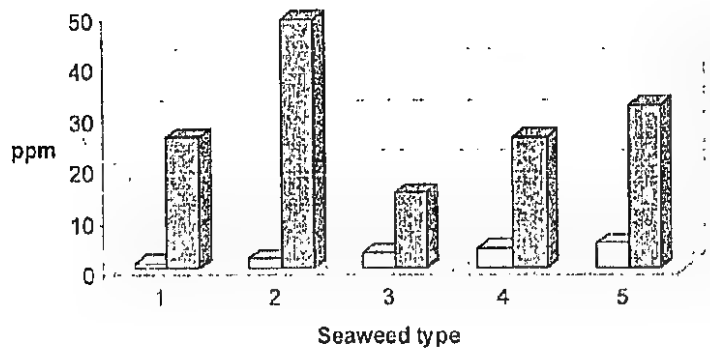
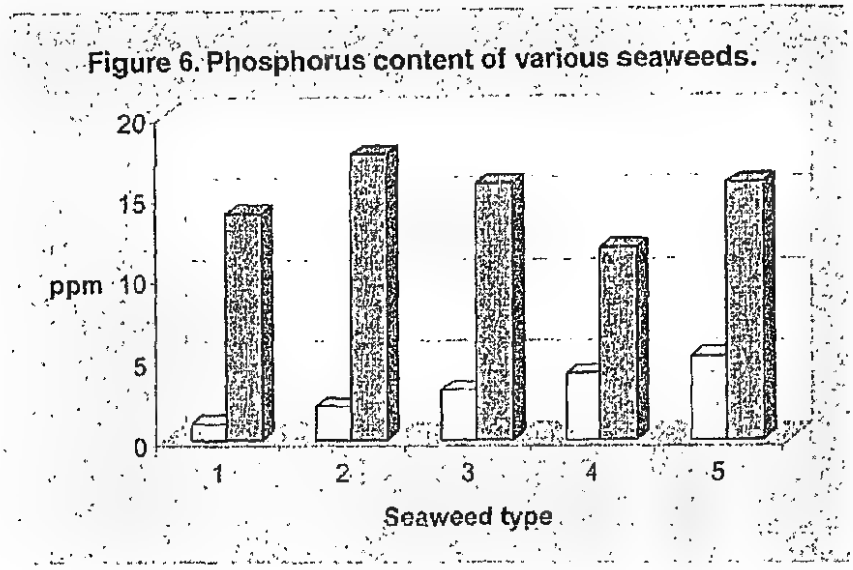


Figure 5. Zinc content of various seaweeds.





flexilis. *S. gigantifolium* was found to contain large amount of calcium and magnesium and sufficient amount of zinc and phosphorus. *S. crassifolium* and S. sp. contained large amount of phosphorus next to *L. flexilis*.

Since *H. durvillaei* and *L. flexilis* contain high levels of iodine, the two samples were further analyzed for their heavy metals content (see Table 4). *H. durvillaei* showed minute amount of mercury, cadmium and lead. However, *L. flexilis* exhibited high level of lead which indicated that it is better for *H. durvillaei* for the formulation studies.

Table 4. Result of heavy Metals analysis.

	Hg (ppm)	Cd (ppm)	Pb (ppm)	As (ppm)
Halymenia durvillaei	0.0055	0.67	1.80	nil
Laurencia flexilis	nil	0.54	3.39	nil

Acute oral toxicity test LD_{50} was determined for *H. durvillaei*. The median lethal dose (LD_{50}) of the sample administered orally in Swiss male mice is 119.1489 ± 4.9873 g/kg. Toxidrome ranged from decreased motor activity, increased respiratory rate, ptosis, writhing, difficulty of breathing and death of mice. Details of acute oral toxicity test is shown in Table 5.

Several formulations of seaweed tablet were tried in order to yield a product that is comparable to the commercial kelp pill. These were based on the US recommended daily allowance of iodine for adults and children twelve years old or more which is 0.15 mg of iodine per tablet. Other parameters which were taken into consideration are tablet hardness, weight variation, disintegration test and dissolution rate test. The final

Table 5. Result of acute oral toxicity test (LD_{50}) in mice.

$$= \frac{\text{Number of mice with positive sign (death)}}{\text{Total number of animals tested}}$$

Group Number	Dose g/kg	n	Number of Deaths				
			Day 1	Day 2	Day 3	Day 7	Day 14
I	0*	10	0/10	0/10	0/10	0/10	0/10
II	109.3	10	4/10	4/10	4/10	4/10	4/10
III	140.32	10	8/10	8/10	8/10	8/10	8/10
IV	180.00	10	10/10	10/10	10/10	10/10	10/10

* Control - same volume as in highest dose

Dose g/kg	n	Observation
0	10	No effect
109.39	10	Immediately after drug administration, the mice manifested decreased motor activity, increased respiratory rate, ptosis, piloerection and difficulty in breathing. Four (4) out of ten (10) mice died within twenty-four (24) hours. The remaining six (6) mice recovered after twenty-four hours.
140.32	10	Immediately after drug administration, the mice manifested decreased motor activity, increased respiratory rate, ptosis, piloerection, writhing, difficulty of breathing followed by death. Eight (8) out of ten (10) mice died within twenty-four (24) hours. The remaining two (2) mice recovered after twenty-four hours.
180	10	Immediately after drug administration, three mice manifested decreased motor activity, increased respiratory rate, ptosis, piloerection, writhing, difficulty in breathing followed by death of all mices within twenty-four (24) hours.

Autopsy findings: Animals which died within twenty-four (24) hours had grossly normal findings while those sacrificed after fourteen (14) days had white spots in the liver.

formulation of the seaweed tablet consisted of 60% dried *H. durvillaei* with 38% of microcrystalline cellulose. Calcium stearate and stearic acid were added each at 1.0% as glidant and binder. The iodine content was about 0.15 mg per tablet with the following minerals: Ca, 5.43 mg; Mg, 30 mg; Fe, 0.02mg and with 0.003 mg each of Zn and P.

The formulated seaweed tablet was evaluated in terms of its physico-chemical properties (refer to Table 6 for the results). Based on the results, the formulated seaweed tablet was comparable to the commercial kelp pill.

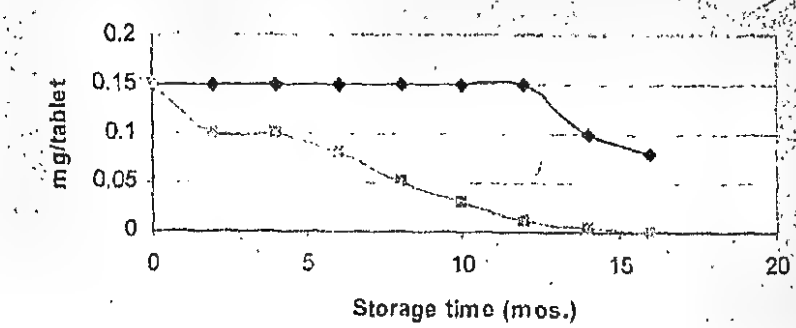
Table 6. Physico-chemical properties of seaweed tablet.

	Formulated Seaweed Tablet	Kelp Pill Commercial
Tablet hardness	3.5 kg	3.0 - 4.0 kg
Weight variations	Meet the requirements for tablet, (USP XIX)	Meet the requirements for tablet, (USP XIX)
Disintegration test	30 minutes	30 minutes
Dissolution rate test 20 minutes (time required for 60% of seaweed tablet to dissolve in the dissolution medium)	comparable results	comparable results

The total microbial count of the formulated seaweed tablets showed the same magnitude as that of the commercial kelp pill which was less than 10 microorganisms per g of sample.

Stability studies showed that seaweed tablet must be kept in a cool, dry place and packed in amber-colored bottles. Results (Figure 9) showed the iodine decomposed rapidly when seaweed tablet is exposed at 40°C , than when exposed at room temperature (Figure 7). Storage at cold temperature (4°C, Figure 8) greatly reduced decomposition of iodine and prolonged the shelf-life of the product than at room temperature.

Figure 7. Effect of room temperature (32°C) on the iodine content of seaweed tablet (exposed/unexposed).



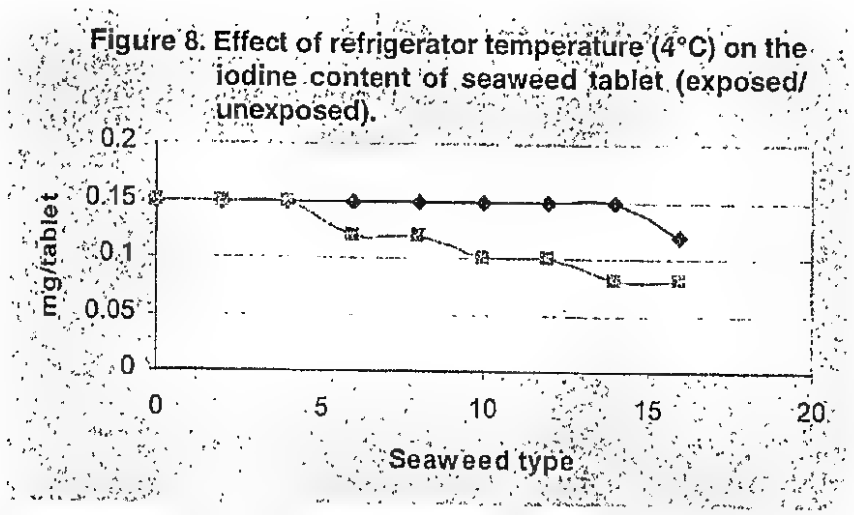


Figure 8. Effect of refrigerator temperature (4°C) on the iodine content of seaweed tablet (exposed/ unexposed).

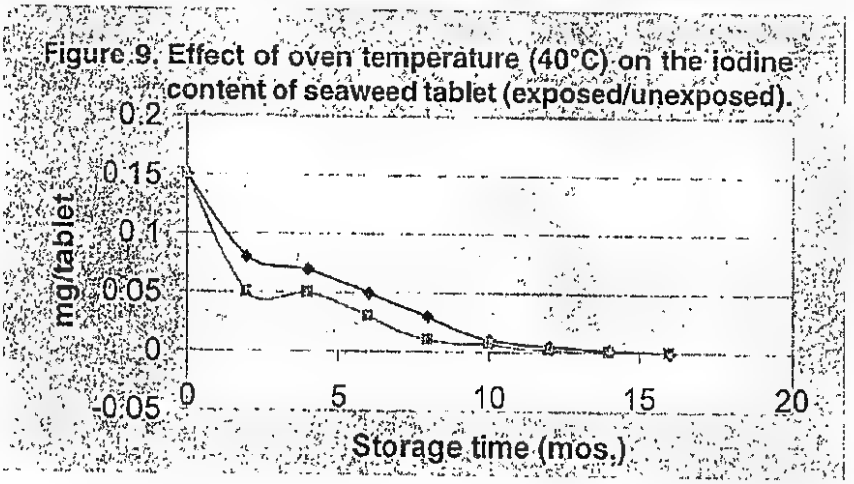


Figure 9. Effect of oven temperature (40°C) on the iodine content of seaweed tablet (exposed/unexposed).

SUMMARY AND CONCLUSION

1. The physico-chemical properties of the formulated seaweed tablet was comparable with those of the commercial kelp pill.
2. The product was found to be a good dietary supplement as source of iodine.
3. The results of the study showed the potential of exploring indigenous materials for commercial production of seaweed tablet as substitute for imported kelp pill.

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Glomus bagyarajii Sp. Nov., A New Species of Glomaceae (Glomales, Zygomycetes) from India

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ABSTRACT

A new species of the arbuscular mycorrhizal fungus, Glomus, isolated from contaminated pot cultures of other glomalean fungi is described. The fungus, named G. bagyarajii, produces abundant bright yellow to yellow brown spores, (36-)100.8(-125) μm, with four wall layers in two groups. Group A consists of an outer, hyaline evanescent wall layer, (0.5-)3.0 (-3.5) μm thick and an inner yellow to yellow brown laminated layer, (1.6-)3.6 (-4.0) μm thick. Group B consists of an outer hyaline unit wall layer, (0.5-)1.0(-1.6) μm thick and an inner hyaline, membranous wall layer, (0.25)0.5(-1.0) μm thick. Reaction of wall layers to Melzers's regent is negative. Subtending hypha hyaline to pale yellow, cylindrical or funnel-shaped (0.7-)10.8(-14.0) μm wide at the spore base. Germination is by regrowth of the subtending hypha.

INTRODUCTION

Glomalean taxonomy is still in the formative stages of exploration and documentation of fungal diversity because very few areas of the world have been extensively sampled for indigenous species (Morton, 1993). Only few *arbuscular mycorrhizal* (AM) species have been described from India (Gerdemann and Bakshi, 1976; Mukherji *et al.*, 1983).

Description of AM fungi is mainly based on the morphological characteristics of spores and sporocarps formed in soil (Morton, 1988; Walker, 1992). Spore wall characteristics are now recognized as the most important criteria in delimiting AM fungal species (Walker, 1983; Morton, 1988; Mehrotra and Baijal, 1994), but are dependent on the clarification of spore development (Walker 1992) and reactions to different mountants and fixatives (Spain, 1990). Studies on the changes in wall characters with spore or sporocarp development may improve definition of these characters (Giovannetti *et al.*, 1991); Franke and Morton, 1994). In addition, it has been found that anomalies in interpretation of spore characteristics can be avoided if colour, wall and germination characteristics are studied first in water and then in other mountants (Spain, 1990; Walker, 1992). Ultrastructural studies of spore

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morphology and the process of spore reproduction may be used to clarify the observations made with light microscope (Maia *et al.*, 1994). Inclusion of certain key characteristics of intraradical structures of arbuscular mycorrhiza, as an additional criteria to identify AM fungi, has also been suggested (Morton and Bentivenga, 1994), but phenotypic features of such structures have been found to be affected by host species (Brundrett and Kendrick, 1990).

The aim of this paper is to describe an unreported species of *Glomus* which produces abundant soil-borne spores in pot cultures. The species was observed as a frequent contaminant in pot cultures of AM fungi isolated from revegetated coal mine spoils.

MATERIALS AND METHODS

Pot Culture

Spores of the undescribed species of *Glomus* were extracted by wet sieving and decanting (Gerdemann and Nicolson, 1963) from contaminated pot cultures of AM fungi isolated from revegetated coal mine spoils. Few morphologically identical spores of the fungus were selected, surface-sterilized and placed singly onto the roots of sterile corn (*Zea mays* L.) seedlings. Inoculated seedlings were regularly observed for spore germination and infection of roots under a dissecting microscope. Infected seedlings were transplanted to autoclaved pots containing sterilized sandy-loam soil and sand mixture (1:1 v/v) as the growth medium. Pots were kept in a greenhouse at 25-30°C with 13 hr photoperiod. Plants were watered daily according to the requirement. Pot cultures were harvested sequentially from 4th to 24th week after transplanting to study the life cycle of the fungus and its spore development.

Measurement of mycorrhizal structures

Pot cultures were finally harvested after 24 weeks. Roots were cleared in 10% (w v) KOH solution at 90°C and stained with 0.05% (w v) acid fuchsin in lacto-glycerol. Stained root pieces were examined under light microscope for measurement of mycorrhizal structures. Spores and sporocarps, extracted from soil were mounted in water, polyvinyl alcohol/ lactic acid glycerol (PVLG, Koske and Tessier, 1983), mixture of PVLG and Melzer's reagent (1:1 v v) and mixture of PVLG and cotton-blue. Spore colour was examined under a dissecting microscope on freshly collected spores immersed in water. Spore and wall dimensions were measured on at least 100 spores mounted in water and PVLG. Classification, wall descriptions and terminology follow those suggested by Morton and Benny (1990), Walker (1983, 1986), Berch and Koske (1986), Morton (1986) and Berch (1987). Spelling of scientific names are those suggested by Almeida (1989).

Voucher specimens have been deposited in the Department of Botany, University of Allahabad, Allahabad and Centre for Mycorrhizal Culture Collection (CMCC), New Delhi, India.

Germination Studies

Spores of the undescribed AM fungal species, kept at 4°C for two months, were surface – sterilized with 0.5% sodium hypochlorite for 3 min, followed by 200 ppm solution of streptomycin and washed three times in sterile distilled water. Spores were then transferred to 1% water agar plates and incubated at 24°C. Germinated spores were stained with cotton blue and observed with light microscope.

Scanning Electron Microscopy

Selected spores of the AM fungus were fixed in 3.0% (w/v) glutaraldehyde in 0.02M phosphate buffer (pH 6) at 4°C for 24 hr, dehydrated in graded ethanol series, mounted on an aluminum stub with colloidal silver paint, coated with gold and examined using a Leica stereoscan 430 scanning electron microscope.

RESULSTS AND DISCUSSION

Species Description

Glomus bagyarajii Mehrotra, sp. Nov. (Figs. 1-8)

Sporocarpia hypogea, sub-globose vel irregularia, (160-320(-480) X (200-) 400(-640) µm. Peridium destitum. Sporae singulae in solum, luteolo vel albolutea, globosae, (36-) 100.8 (-125.2) µm dia, sub-globosae vel irregulares, (56-) 108 (-126) X (49-) 90 (-104.4) µm efformatae. Tunica sporae e stratis quatuor in turmis duabus. Turma externa (Group A) e stratis duabus (strata 1 & 2); uno evanescenti, hyalino, (0.5-) 3.0 (-3.5) µm crasso; secundo laminato, fulvo ad aureobruneam, (1.6-) 3.6 (-4.0) µm crasso; turma interior (Group B) e stratis duabus (strata 3 & 4); tertio rigido, hyalino, (0.5-) 1.0 (-1.6) µm crasso; quarto membranaceo, hyalino, (0.25-) 0.5(-1.0) µm crasso. Hyphae sustenantes rectae vel curvatae, cylindricae vel infundibuliformis, (7.0-) 10.8 (-14.0) µm dia. Formans arbusculare mycorrhizae.

Sporocarps hypogeous, sub-globose to irregular, (160-) 320(-480) X (200-) 400(-640) µm. Peridium absent. Chlamydospores formed singly in soil, yellow to yellow brown in transmitted light, globose, (36-) 100.8(-125.2) µm dia, sub-globose to irregular-shaped, (56-) 108 (-126) X (49-) 90 (-104.4) µm. Spore lumen content with many small lipid globules.

Spore wall structure of four layers in 2 groups (A&B). Group A: wall layer 1, evanescent, hyaline, (0.5-) 3.0 (-3.5) µm thick, closely adherent to wall layer 2 in young spores, but separates in mature spores; wall layer 2, laminated, yellow to yellow brown, with 3-4 laminae, (1.6-) 3.6(-4.0) µm

thick, generally thicker near the point of attachment. Group B: wall layer 3 unit, hyaline , (0.5-) 0.1(-1.6) µm thick; wall layer 4 membranous, hyaline, (0.25-) 0.5(-1.0) µm thick.

Subtending hypha hyaline to pale yellow, straight or recurved, cylindrical of funnel-shaped, (7.0-) 10.8 (-14 0) µm wide at the spore base. Wall of subtending hypha 1-1.5 µm thick, continuous with spore layers 2 and 3.

Pore at the spore base, 2-3 6 um wide, occluded by curved septum or by the innermost membranous wall layer. Reaction of wall layers to Melzer's reagent negative Wall layer 1,3 & 4 can be easily detected when stained with cotton-blue.

Distribution and habitat

To date, G. *bagyarajii*, is known from India. Origin of spores of G. *bagyarajii* is unknown Chemical properties of the soil were pH-7.7; available P- 4.5 mg kg; OC-0 88%; K-25.8 mg/kg.

Mycorrhizal associations

G bagyaraju formed arbuscular mycorrhizal associations in pot cultures with *Zea mays* L., *Leucaena leucocephala* (Lamk.) de Wit and *Cenchrus ciliaris* L. This species also formed intraradical spores.

Etymology

Named after D J. Bagyarajii in recognition of his contributions to the field of mycorrhizal research.

Specimens examined

Holotype – India. Department of Botany, University of Allahabad, Allahabad. From pot cultures (Culture No. SS-16) on Zea mays L. Isotype: India. Deposited in Centre for Mycorrhizal Culture Collection (CMCC), Tata Energy Research Institute, New Delhi, India (Culture No. AM-1019).

Mycorrhizal Formation and Morphology

One month after transplanting, mycorrhizal infection was 55% in corn plants. Intraradical hyphae were hyaline, (3.5-)5.4(-7.2) µm. Spores formed inside the roots were globose, subglobose to irregular-shaped, (72-) 108 X 42 (-57.6) µm Extramatrical hyphae were hyaline to pale yellow, (3.5-) 7.2(-10.8) µm wide. Two months after transplanting, sporocarps were produced in pot cultures (Fig. 1). Spores in sporocarps were observed in three stages of development: (i) very young spores were hyaline, with two wall layers: wall layer 1, evanescent, roughened, 0.25-0 5 um thick (Fig 3) and wall layer 2 unit, smooth, 0 5-1 0 µm thick, (ii) young spores were pale yellow to yellow, with three wall layers: wall layer 1 hyaline, evanescent, 0.5-2.0 µm thick; wall layer 2 laminated, 1.6-3.0 µm thick and wall layer 3 unit, 0.25-0.5 µm thick, which may

Figures 1-3. Scanning electron micrographs of Glomus bagyarajii.

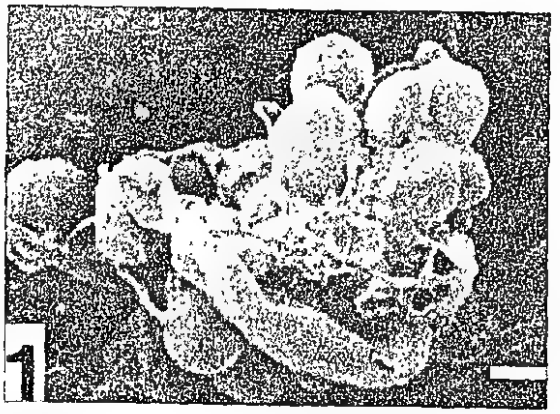

Figure 1. A sporocarp. Scale bar = 54 µm.

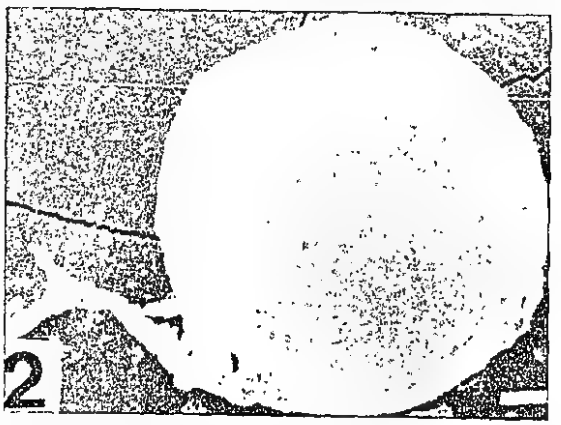

Figure 2. A spore with funnel-shaped hyphal attachment. Scale bar = 26 µm.

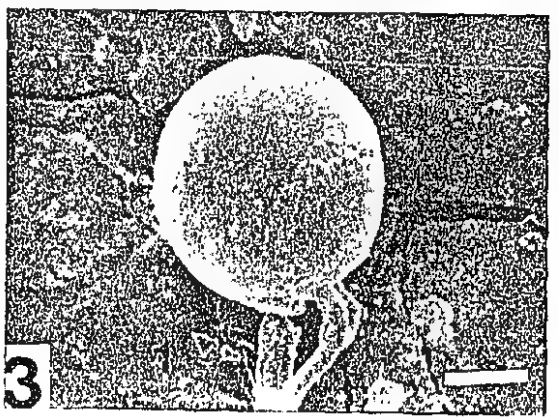

Figure 3. A young spore with sloughed off wall layer (arrow). Note the roughness of the evanescent layer. Scale bar = 26 µm.

sometimes appear as membranous layer (Fig.5); (iii) mature spores were yellow to yellow brown, with four distinct wall layers: wall layer 1 evanescent, 2.5-3.0 µm thick (Fig. 4); wall layer 2 laminated, 3-3.6 µm thick; wall layer 3 unit, 0.5-1.0 µm thick; wall layer 4 membranous, 0.25-0.5 µm thick (Fig 6 & 8). In *Gigaspora* spp., where direct spore

Figures 4-8. Light micrographs of the spore wall structure of G. bagyarajii.

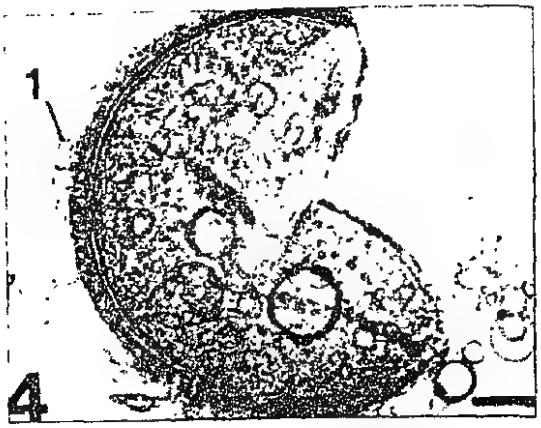

Figure 4. A mature spore with remnants of the outermost wall layer (1). Scale bar = 30 µm.

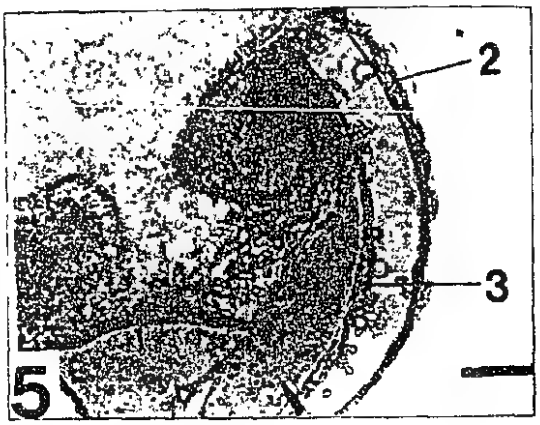

Figure 5. A crushed spore revealing the laminated (2) and unit (3) wall layers. Scale bar = 20 µm.

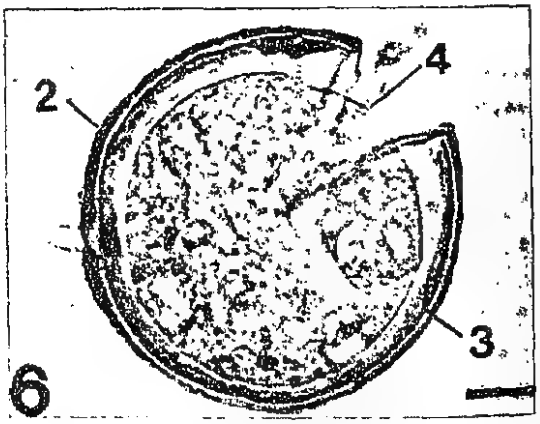

Figure 6. A spore with laminated (2), unit (3) and membranous (4) wall layers. Scale bar = 20 µm.

germination like *Glomus* spp. occurs. spore development proceeds in a linear sequence divided into 3 stages: stage 1 - initial spore expansion coupled with differentiation of two thin layers of almost equal thickness; stage 2 - laminae added to the innermost layer; stage 3 – papillate layer differentiated on the inner surface of the laminae followed by the

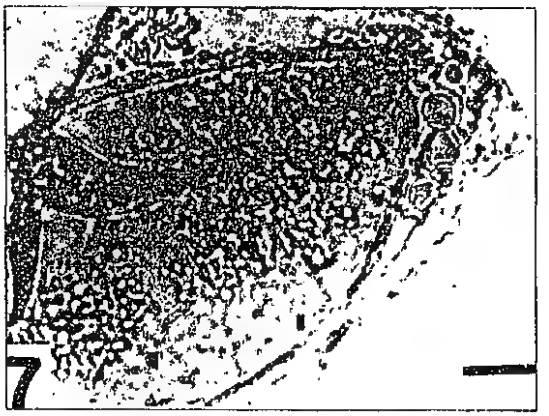

Figure 7. A ruptured spore wall with evanescent, laminated and unit wall layers. Scale bar = 20 µm.

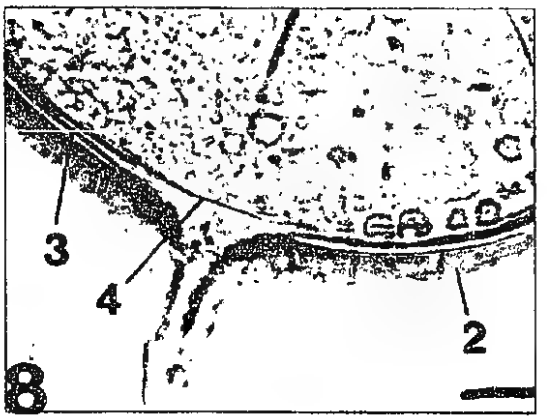

Figure 8. A mature spore with laminated (2), unit (3) and membranous (4) wall layers. Note the occlusion of the pore at the point of hyphal attachment.

emergence of germ tube (Bentivenga and Morton, 1995). From the present study, it appears that the wall differentiation in *Glomus* spp. also takes place in three stages of spore development.

Very young spores of *G. bagyarajii* were hyaline, as has also been observed for spores of *G. ambisporum* Smith & Schenck (Smith & Schenck, 1985), *G. coronatum* Giovannetti, Avio & Salutini (Giovannetti *et al.*, 1991), *G. intraradix* Schenck & Smith (Chabot *et al.*, 1992) and *Entrophospora kentinensis* (Wu *et al.*, 1995). This indicates that most *Glomus* spp., if not all (e.g., *G fasciculatum*, Walker & Koske, 1987), follow the same sequence of change in spore colour during early stages of spores development. It appears that the age of the spore is of decisive significance to the value of spore colour as a character for identification of AM fungal species.

Germination in *G. bagyarajii* occurred by the regrowth of the subtending hypha, as has also been reported in most Glomus spp. (Sequeira et al., 1985); Meier and Charvat, 1992; Giovannetti et al., 1991).

Ultrastructural study of G. bagyarajii spores showed that: (i) shape of the

subtending hypha near the spore base is either cylindrical or funnel-shaped; (ii) the outermost evanescent wall layer is roughened; and (iii) no ornamentation is present on the spore surface.

Spores of G. bagyarajii can be distinguished from other Glomus spp. by their bright yellow to yellow brown colour. Young spores of *G. bagyarajii* resemble those formed by *G. fasciculatum* (Thaxter) Gerd & Trappe emend. Walker & Koske in having yellow spores and 3 wall layers. Spores of G. bagyarajii, however, possess an outermost evanescent wall layer and the wall layers do not stain Melzer's reagent. In addition, mature spores of *G. bagyarajii* possess 4 wall layers. Young spores of G. bagyarajii might also be confused with those of *G. etunicatum*, particularly when the innermost wall layer in the former is closely adherent to the laminated layer. However, the two species can be readily distinguished by the presence of 4 wall layers in mature spores of *G. bagyarajii.* The spore colour and wall characteristics of *G. bagyarajii* are almost similar to that of *G. manihot* Howeler, Sieverding & Schenck, but differs in having: (i) sporocarp; (ii) smaller spore diameter; and (iii) innermost membranous wall layer.

This and other studies (Franke and Morton, 1994; Giovannetti et al., 1991; Bentivenga and Morton, 1995) have shown that within a composite spore wall, phenotypically distinct layers are formed during the process of spore formation. Additional studies on the morphological and ontogenetic events in spores are required for improving the use of wall characteristics in identification of AM fungi.

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Antifungal Flavonoids from Waltheria americana

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ABSTRACT

The chloroform extract of Waltheria americana afforded 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone (1) and 5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone (2). Their structures were elucidated by extensive 1D and 2D NMR techniques. Compound 1 showed high antifungal activity against C. albicans and low activity against T. mentagrophytes, while 2 showed moderate antifungal activity against A. niger and T. mentagrophytes. Compound 1 has the same activity as chlotrimazole at a concentration of 30 μg against C. albicans.

Keywords: *Walteria americana*, Sterculiaceae, oxyanin A, 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone, 5,2'-dihydroxy-3,7,4'5'-tetramethoxyflavone, antifungal

INTRODUCTION

Waltheria americana [common name: Barulad (Ilocano) and Kanding-kanding (Negros Occidental)] belongs to the family Sterculiaceae. It is a common weed found throughout the Philippines. The plant is used as a febrifuge, a mucilaginous, an antisyphilis, a purgative and an astringent [1]. Chemical investigations of the plant afforded peptide alkaloids [2], alkaloids [3], flavonoids, flavonoid glycosides and caffeic acid [4]. We now report the isolation, structure elucidation and antifungal activity of 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone (**1**) and 5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone (**2**). Compound **1** (common name: oxyanin A) was reported to have been isolated from the heartwood of *Distemonanthis benthamianus* and *Apuleia leiokarpa* [5-7]. This is the first report on the isolation of **1** and **2** from *Waltheria americana* and the structure elucidation of **1** by 1D and 2D NMR spectroscopy. A previous study reported that the glucoside of **2** occur naturally in *Chrysosplenium americanum* [8]. Related literature revealed that flavonoids of similar structures have antifungal activity [9-10].

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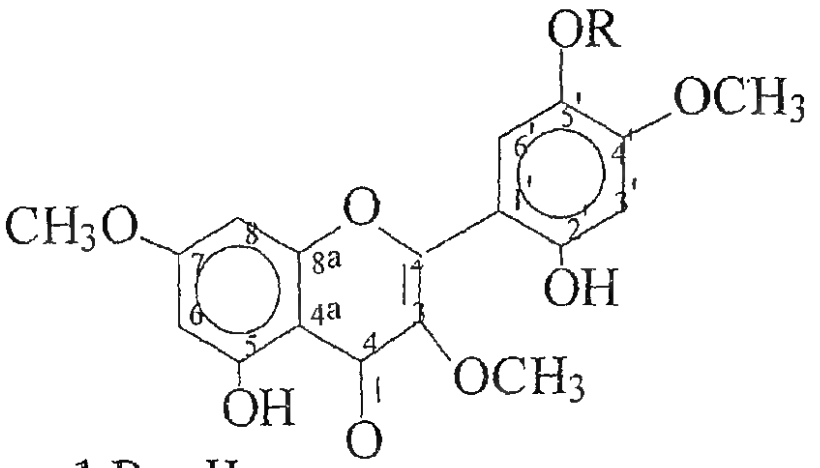


1 R = H

2 R = CH_3

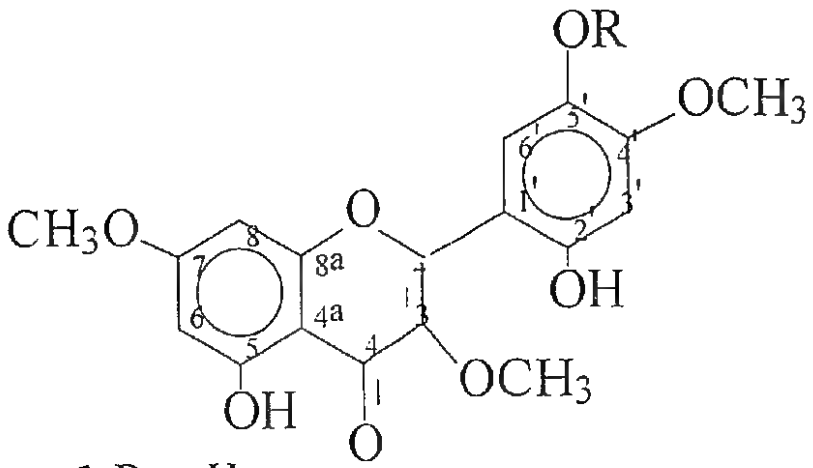
RESULTS AND DISCUSSION

The 10% Me_2CO in $CHCl_3$ fraction was rechromatographed in 20% Me_2CO in $CHCl_3$ to afford 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone (**1**) and 5,2'-dihydroxy-3,7,4'5'-trimethoxyflavone (2) after recrystallization from diethyl ether. The structure of **1** was elucidated by extensive 1D and 2D NMR techniques, while the structure of **2** was deduced by comparison of its ^1H and ^{13}C NMR spectral data with those of **1**.

Compound **1** is an orange needle with a melting point of 229-231 °C. The mass spectrum of **1** gave a molecular ion peak at $M^+ = 360$ which corresponds to a molecular formula of $C_{18}H_{16}O_8$. The ^1H NMR spectrum (Table 1) indicated resonances for aromatic protons at δ 6.38 (1H, d, 2.15), δ 6.46 (1H, d, 2.15), δ 6.61 (1H, s) and 7.22 (1H, s); two methoxy groups at δ 3.88 (6H, s) and one at δ 3.95 (3H, s); three hydroxyl groups at δ 5.38 (1H, s), δ 7.88 (1H, s) and δ 12.39 (1H, s) which were verified by D_2O exchange experiments.

Table 1. ^1H NMR spectral data of 1 and 2 in $CDCl_3$ (300 MHz).

H	1, δ	2, δ
H-6	6.38 (1H, 2.15 Hz)	6.40 (1H, 2.15 Hz)
H-8	6.46 (1H, 2.15 Hz)	6.44 (1H, 2.15 Hz)
H-3'	7.22 (1H, s)	7.12 (1H, s)
H-6'	6.61 (1H, s)	6.61 (1H, s)
5-OH	12.39 (1H, s)	12.41 (1H, s)
2'-OH	7.88 (1H, s)	7.90 (1H, s)
5'-OH	5.38 (1H, s)	
5'-OCH3		3.92 (3H, s)
3-OCH3, 7-OCH3	3.90 (6H, s)	3.88 (6H, s)
4'-OCH3	3.95 (3H, s)	3.92 (3H, s)



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5'-OCH3		3.92 (3H, s)
3-OCH3, 7-OCH3	3.90 (6H, s)	3.88 (6H, s)
4'-OCH3	3.95 (3H, s)	3.92 (3H, s)

The ^{13}C NMR spectrum of **1** (Table 2) showed resonances for eighteen carbons with the following functionalities: a carbonyl carbon at δ 177.4, eight oxygenated aromatic carbons at δ 165.9, δ 162.0, δ 157.4, δ 155.9, δ 151.0, δ 150.4, δ 139.9 and δ 136.8; six aromatic carbons at δ 113.0, δ 109.8, δ 105.9, δ 102.2, δ 98.3 and δ 92.5; and three methoxy groups at δ 62.2, δ 56.2 and δ 55.9.

Table 2. ^{13}C NMR spectral data of 1 and 2 in $CDCl_3$ (75 MHz).

^{13}C	1, δ	2, δ
C-2	155.9	155.9
C-3	136.8	136.7
C-4	177.4	177.3
C-4a	105.9	105.8
C-5	162.0	162.0
C-6	98.3	98.2
C-7	165.9	165.8
C-8	92.5	92.5
C-8a	157.4	157.3
C-1'	109.8	108.6
C-2'	150.4	151.3
C-3'	102.2	102.9
C-4'	151.0	153.9
C-5'	139.9	143.6
C-6'	113.0	110.8
3-OCH3	55.9	55.9
7-OCH3	62.2	62.2
4'-OCH3	56.2	56.1
5'-OCH3		56.7

The ^{13}C and ^{1}H assignments were verified by HMQC and connectivity was verified by inverse long-range heteronuclear experiment HMBC optimized for J = 10 Hz (Table 3). All long-range correlations observed were consistent with the proposed structure of **1**. The structure of **1** was confirmed by NOESY which indicated correlation through space of the ^{1}H nuclei in the molecule (Fig. 1). In addition, the melting point of **1** is similar to the melting point of 5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone [11].

Compound **2** is a yellow crystal with a melting point of 119-121 °C. The structure of **2** was deduced by comparison of its ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) spectral data with those of **1**. The ^{1}H NMR spectrum of **2** indicated the loss of the hydroxyl proton at δ 5.38 (1H, s) in **1** and the appearance of an additional methoxy group at δ 3.92 (3H,s) in **2**. The ^{13}C NMR spectrum of **1** showed an additional methoxy carbon at δ 56.7. The structure of **2** was confirmed by the mass spectrum which gave a molecular ion peak [M$^+$] = 372 corresponding to a molecular formula of $C_{19}H_{18}O_8$.

Table 3. ^{13}C-^{1}H long-range correlation of 1.

Protons, δ	long-range het. corr. expt.
6.40 (H-6)	95.5 (C-8), 105.9 (C-4a), 165.9 (C-7)
6.44 (H-8)	98.3 (C-6), 105.9 (C-4a), 157.4 (C-8a), 165.9 (C-7)
6.60 (H-3')	139.9 (C-5'), 151.0 (C-4')
7.22 (H-6')	139.9 (C-5'), 150.4 (C-2'), 151.0 (C-4')
12.39 (5-OH)	162.0 (C-5)
7.88 (2'-OH)	102.2 (C-3'), 150.4 (C-2')
5.40 (5'-OH)	113.0 (C-6'), 139.9 (C-5'), 151.0 (C-4')
3.90 (3-OCH3)	136.8 (C-3)
3.90 (7-OCH3)	165.9 (C-7)
3.95 (4'-OCH3)	151.0 (C-4')

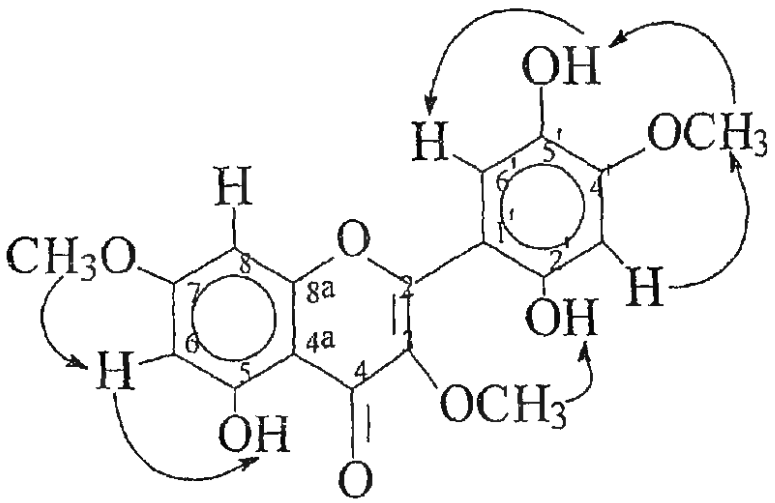

Figure 1. NOESY correlation of 1.

Compound 1 was tested for antimicrobial activity against seven microorganisms, namely: *E. coli, P. earuginosa, S. aureus, S. typhi, C. albicans, A. niger* and *T. mentagrophytes*. Results of the study (Table 4) indicated that 1 is active against a parasitic fungus, *T. mentagrophytes* at concentrations of 5, 10 and 15 μg with antimicrobial index (AI) of 0.2, 0.4 and 0.4, respectively. It was also found to be active against a yeast like organism, *C. albicans* with AI of 0.2, 0.5 and 2.0 at concentrations of 20, 25 and 30 μg, respectively which is the same as the activity of a commercial drug, clotrimazole. *T. mentagrophytes* is a parasitic fungus that lives in the skin and causes various dermatomycoses and ringworm [12], while *C. albicans* is found on the mucous membranes, intestinal tract, vagina and skin [13]. Results of the study imply that 1 can be used to prevent the proliferation of the tissues of any infections caused by these fungi. Compound 1 was found to be inactive against *A. niger* at

concentrations of 5, 10 and 15 μg. The lesser concentrations of **1** compared to the antibiotics used in the test against *T. mentagrophytes* and *A. niger* are due to an earlier study [14] which reported that a mixture of **1** and **2** from the plant extract showed very high activity against these two fungi, even higher than the standard antibiotics. The study also showed that **1** is inactive against the gram-positive bacteria: *S. aureus* and *S. typhi* and the gram negative bacteria: *E. coli* and *P. aeruginosa*.

Table 4. Antimicrobial test results of 1.

Test Organism	Sample	Amt. of Sample, μg	Clearing Zone, mm 1	Clearing Zone, mm Antibiotic	AI
S. aureus	1	20	-	-	-
		25	-	-	-
		30	-	-	-
	chloramphenicol	30		30	2.0
E. coli	1	20	-		-
		25	-		-
		30	-		-
	tetracycline	30		40	3.0
A. niger	1	5	-		-
		10	-		-
		15	-		-
	cyclohiximide	30		15	0.5
T. mentagophytes	1	5	12		0.2
		10	14		0.4
		15	14		0.4
	chlotrimazole	30		62	5.2
P. aeruginosa	1	20	-		-
		25	-		-
		30	-		-
	tetracycline	30		28	1.8
S. typhi	1	20	-		-
		25	-		-
		30	-		-
	chloramphenicol	30		23	1.3
C. albicans	1	20	12		0.2
		25	15		0.5
		30	30		2.0
	chlotrimazole	30		30	2.0

Compound **2** was tested for antimicrobial activity against the same microorganisms used against **1** (Table 5). It was found to be active against *T. mentagrophytes* with AI of 0.3, 1.3, 1.8, and 1.8 at concentrations of 15, 20, 25 and 30 μg, respectively. It was also found to be active against *A. niger* with AI of 0.1 and 0.3 at concentrations of 25 and 30 μg, respectively. *A. niger* is a multicellular mold that poses as a

perennial contaminant and causes aspergillosis disease [13]. Thus, **2** can be used to prevent the proliferation of the tissues of any infections caused by this mold. Compound **2** was inactive against the other microorganisms tested.

Table 5. Antimicrobial test results of 2.

Test Organism	Sample	Amt. of Sample, µg	Clearing Zone, mm		AI
			1	Antibiotic	
S. aureus	2	20	-	-	-
		25	-	-	-
		30	-	-	-
	chloramphenicol	30		30	2.0
E. coli	2	20	-		-
		25	-		-
		30	-		-
	tetracycline	30		40	3.0
A. niger	2	5	-		-
		10	-		-
		15	-		-
		20	-		-
		25	11		0.1
		30	13		0.3
	cyclohiximide	30		15	0.5
T. mentagophytes	2	5	-		-
		10	-		-
		15	13		0.3
		20	23		1.3
		25	28		1.8
		30	28		1.8
	chlotrimazole	30		62	5.2
P. aeruginosa	2	20	-		-
		25	-		-
		30	-		-
	tetracycline	30		28	1.8
S. typhi	2	20	-		-
		25	-		-
		30	-		-
	chloramphenicol	30		23	1.3
C. albicans	2	20	-		-
		25	-		-
		30	-		-
	chlotrimazole	30		30	2.0

EXPERIMENTAL

General Experimental Procedures

NMR spectra were recorded in $CDCl_3$ solutions on a Bruker AMX 300 nmr spectrometer with $CDCl_3$ (δ 7.26, 77.0 ppm) as reference. The low resolution EIMS was carried out on a *JEOL D 100* mass spectrometer. Silica gel type 60 (Merck) was used for column chromatography and plastic-backed plates coated with Silica gel F254 (Merck) for thin layer chromatography. Plates were visualized by spraying with vanillin/H_2SO_4 and warming.

Sample Collection and Isolation

Waltheria americana was collected from Sinait, Ilocos Sur in February 1996. The sample was identified at the Philippine National Herbarium of the National Museum. The air dried leaves (200 g) were soaked in chloroform (800 ml), then filtered. The filtrate was concentrated *in vacuo* to afford a crude extract (14 g). The crude extract was treated with aqueous $Pb(OAc)_2$ to precipitate the pigments [15]. The treated extract (3.0 g) was subjected to gravity column chromatography packed with silica gel G (60-230 mesh) and eluted with increasing proportions of acetone in chloroform (10% increment). The 30% acetone in chloroform fraction afforded a mixture of **1** and **2** (0.1 g) after recrystallization in Et_2O. This was rechromatographed in 20% acetone in chloroform to afford **1** (10 mg) and **2** (16 mg) after recrystallization from diethyl ether.

Bioassay: Antimicrobial Tests

The microorganisms used in these tests are *Staphylococcus aureus* UPCC 143, *Escherichia coli* UPCC 195, *Pseudomonas aeruginosa* UPCC 244, *Candida albicans* UPCC 2168 *Aspergillus niger* UPCC 4063, *Trichophyton mentagrophytes* UPCC 4193 and Salmonella typhi UPCC.

Microbial suspension containing approximately 10^7 cells/mL was prepared for each test organism for 24-hour agar culture using 0.1% peptone water. One-tenth (0.1 mL) of the bacterial suspension was transferred into pre-poured 30 mL deep nutrient agar plate, the yeast suspension into glucose yeast peptone agar plate and the fungal suspension on potato dextrose agar plate. About 5 mL of the corresponding melted agar cooled to about 45°C was immediately poured into the plate. The plate was swirled to distribute the microbial cells evenly on the plate. After the overlay agar has solidified, three 1-cm diameter holes were bored from equidistant points using sterile cork borer.

One-tenth (0.1 mL) portions of the compounds were placed in duplicate holes per organism. A similar volume of the solvent acetone and of the corresponding antibiotic for each test organism was placed in the remaining two wells on the plate. Plates were incubated at room temperature to prevent evaporation of liquid on the petri lid that may

cause interference in distribution of organisms on the surface. Bacterial and yeast plates were read after 24 hours, while the mold plate was read after three days. Clearing zones were measured in millimeters (mm), the average for each compound was taken and the antimicrobial activity index (AI) was computed as the clearing zone minus the diameter of the hole divided by the diameter of the hole.

ACKNOWLEDGMENTS

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Growth Rate, Yield and Economics of Gracilariopsis bailinae (Gracilariales, Rhodophyta) Using Fixed Bottom Long-Line Method

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ABSTRACT

Vegetative thalli of Gracilariopsis bailinae weighing 10 g each were tied to a 5-m monofilament line with plastic strips and laid horizontally on the substrate and were observed to grow at 30 d interval for 9 months. The monthly growth rate and yield were determined and a cost and return analysis of the culture system was made. The monthly growth rate of the seaweed was significantly different (P=0.05) over culture month. The highest average growth rate was 6.7% day^{-1} while the lowest was 1.7%. The lowest and highest average yield (dry wt) was 72 g and 660 g m^{-2} mo^{-1}, respectively.

A capital asset of P1,680, working capital of P2,980, and annual production cost of P5,860 were calculated from the culture system. An annual net returns of P31,292 was computed based on a 1,000 m^2 area. Return on investment is 671.50% while payback period is 1.7 months.

INTRODUCTION

Gracilariopsis bailinae like other species of *Gracilaria* is a potential source of agar (Hurtado-Ponce and Umezaki, 1988; Luhan, 1992; Hurtado-Ponce, 1992a). Its production from small-scale farming (Hurtado-Ponce *et al.*, 1992a), wild stock gathering for commercial purposes (Hurtado-Ponce *et al.*, 1992b), and its abundance (de Castro *et al.*, 1991) in Western Visayas are motivating factors to further develop the market value of this agarophyte.

Results from experimental studies show that cultivation of *Gracilariopsis* in cages, both monoculture (Hurtado-Ponce, 1990; Guanzon and de Castro, 1992) and polyculture (Hurtado-Ponce, 1992b) have been encouraging. Still, experiments with larger scale cultivation in mudflats, drainage canals and estuaries are important to determine its economic feasibility. The *Eucheuma* fixed-bottom long line

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technique of Doty (1973) has been adopted in the present study because it is cost effective. This paper describes the growth rate, yield and economics of fixed-bottom long line cultivation of *Gracilariopsis bailinae* in an estuary.

MATERIALS AND METHODS

The study was conducted within a *Gracilaria* farm near an estuary at Leganes, Iloilo. Vegetative thalli of *Gracilariopsis bailinae* (= *Gracilariopsis heteroclada* Zhang *et* Xia), approximately 10 g each, were tied to a 5-m monofilament line (#110) with 'tie-tie' at intervals of 10 cm. Nine cultivation lines arranged in parallel to the current were laid horizontally at 25 cm apart and tied to mangrove posts driven into the bottom. A 30-day growth period was observed for nine months. Every month, all seaweeds were harvested, washed, cleaned, freed of epiphytes and finally weighed on a top loading weighing scale. The original weight of the seedstock was returned with seedlings coming from a selection of the harvest. Mean daily growth rate in each one-month culture period was then calculated according to Rueness and Tananger (1984).

Some environmental parameters like water temperature and salinity were monitored every sampling time using an ordinary Celcius thermometer and Atago refractometer, respectively, while water samples within the cultivation area were collected monthly for pH, NH_3-N, NO_2-N and PO_4-P analyses following the methodology described by Strickland and Parsons (1972).

Cost and returns analysis based on 20% moisture content was used to determine return on investment (ROI) and payback period. ROI was computed by dividing annual net income by the investment. The payback period was computed by dividing the investment by the sum of the annual income and annual depreciation (Shang, 1976). Production costs were seed plants, plastic straw (tie-tie), mangrove post, hired labor, and depreciation. Depreciation was computed using the straight line method based on the estimated useful life of the asset (Meade, 1989). Net income was derived by deducting production costs from gross returns. Costs and returns are based on a 0.1 ha area and the currency used in the computations is the Philippine Peso (P35 = US $1). A partial budget for 'rice planting' and fixed-bottom long line was included to compare the marginal cost, marginal revenue and net benefit. Marginal revenue was computed as the difference of 'rice planting' revenue from fixed-bottom line cultivation; marginal cost was computed as the difference of the total cost of the methods; and net benefit was computed as the difference of marginal cost from marginal revenue.

RESULTS AND DISCUSSION

Line farming

The average monthly specific growth rate (SGR) (Fig. 1) of *G. bailinae* expressed as % increase in wet weight day^{-1} ranged from 1.7 to 6.7% day^{-1} while the yield ranged from 72 to 660 dwt g m^{-2} and these were significantly different over time (culture month) (P 0.05). The lowest SGR was recorded in March while the highest was in November. A decreasing yield was recorded for the first two months of culture, however, an increasing yield was distinct from March reaching its peak in June which gradually declined in August. A sudden increase was recorded in September. Due to successive typhoons in October, the study was terminated on this month. No data was recorded for the month of February due to the adverse effect of pesticide coming from the neighboring ponds.

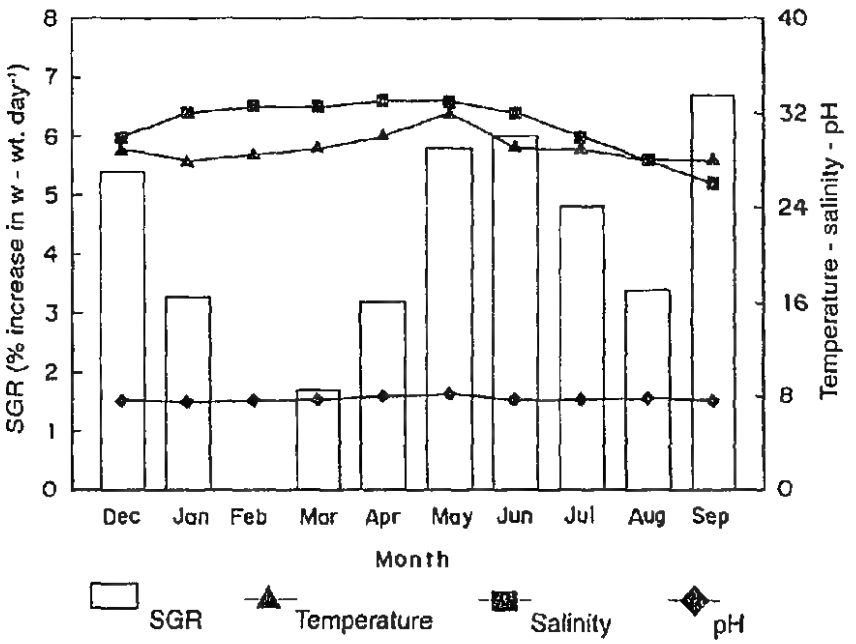

Figure 1. Average monthly specific growth rate (SGR) (% increase in wet wt. day^{-1}) of *G. bailinae* as influenced by temperature, salinity and pH.

Gracilariopsis bailinae when grown horizontally on lines with 'tie-tie' method varied according to culture month just like other species of *Gracilaria* (Wang *et al.*, 1984, Li *et al.*, 1984, Westermeier *et al.*, 1993). An average monthly growth rate of 4.5% day^{-1} was recorded in the present study which is comparable with the results obtained by Camara Netto (1987) on *Gracilaria* (4% day^{-1}) using the same cultivation technique. However, when *G. bailinae* was grown vertically

at different depth levels below the water surface with juveniles of sea bass in floating net cages (Hurtado-Ponce, 1992b), the seaweed demonstrated a much higher growth rate (13.3% day^{-1}). A similar observation was reported in *Gracilaria verrucosa* by Ren *et al.*, (1984) using floating raft (12% day^{-1}) and fixed raft (10% day^{-1}). Better growth of seaweed is usually obtained near the water surface (Kim and Humm, 1965). The adoption of a culture technique to suit the prevailing characteristics of the study site plays a major role in seaweed cultivation. The choice to adopt the fixed-bottom long line cultivation of G. *bailinae* in this study was mainly due to the water level during the lowest tide. In the study site, the area was prepared into fishpond-like with dike elevation of only 30 cm. This was done to allow approximately 25-30 cm of water to remain during the lowest tide in order to avoid direct exposure to the sun and total dessication. As a result, the seaweeds were exposed to constant change of seawater brought by daily tidal changes.

Environmental parameters

Water temperature ranged from 28 to 32°C, salinity from 26 to 33, and pH from 7.5 to 8.2. Lowest water temperature was recorded in January, August and September (28°C) while the highest in May (32°C). The lowest salinity of 26 coincided with the lowest temperature. There was little variation in pH, NO_2-N, NH_3-N and PO_4-P readings during the entire cultivation period. Highest yield coincided with the lowest NO_2-N, NH_3-N and PO_4-P readings (Fig. 2).

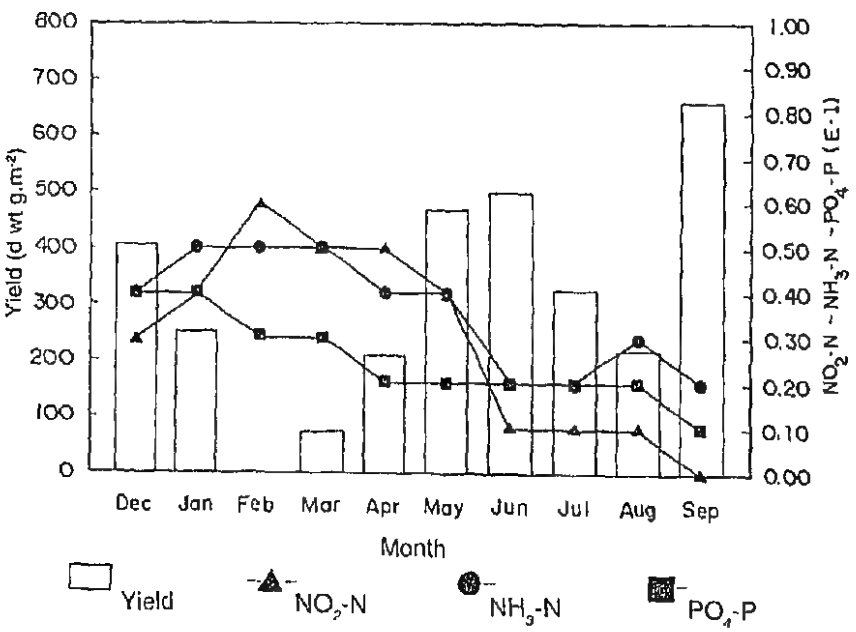

Figure 2. Average monthly yield (dwt g m^{-2}) of *G. bailinae* as influenced by nitrogen-phosphorous contents.

The experimental farm experienced a lot of grazing especially during the juvenile stage (May-June) of siganids and mullets which contributed to the loss of biomass and low growth rate. Similar observations were reported in earlier studies (Doty, 1986, Nelson and Tsutsui, 1981, and Hurtado-Ponce, 1990). Associated algae like *Polysiphonia, Hypnea, Rhizoclonium, Ulva*, and *Enteromorpha* were observed to appear during higher temperature (Shang, 1976; Chiang, 1981). Manual removal of these algae was done to reduce competition in space, nutrients, and sunlight with the cultured species. Highest growth and yield coincided with lower water temperature and salinity. pH readings were within the favorable range (Liu, 1987).

Costs and Returns

Total investment for a 0.1 ha *Gracilariopsis* line farm amounting to P 4,660.00 included cash outlays for capital assets (P1,680) and working capital (P2,980.00) for the first cropping. (Table 1). The cost of production for a 0.1 ha G. *bailinae* line farm was P 5,860.00 yr^{-1}, with 9 crops yr^{-1} (Table 2). At the current farm gate price of P12 kg^{-1} (dry), gross returns crop-1 was P 33,024 (Table 3). A net income of P 31,292 yr^{-1} was computed with an ROI of 671.5% using the line method. Initial investment is recovered in almost two (2) months. *Gracilariopsis bailinae* planted with a fixed-bottom long line method compared with 'rice planting' yields a higher marginal revenue (P23,112), marginal cost (P3,522.24) and net benefit (P19,589.76) (Table 4) which simply indicates that the former method of planting is more productive than 'rice planting' on the same planting area.

Table 1. Investment for a 0.1 ha *Gracilariopsis bailinae* farm using the fixed-bottom long line (US$=35 PhP).

Item	Quantity	Unit Cost	Total Cost	Eco. Life (Year)	Annual Depreciation*
Capital Asset					
Drying platform (bamboo)	1 unit	1,000	1,000	2	500
Polyethylene rope (No.7)	4 rolls	70	280	5	56
Basins/pails (big)	1 pc	400	400	2	200
Sub-total			1,680		756
Working Capital (first crop)			2,980		
Total Investment			4,660		

*Computed using the straight-line method by dividing cost of asset. Value of the asset as scrap is assumed to be zero.

Table 2. Costs of *G. bailinae* cultivation using the fixed-bottom long line method (0.1 ha)[1].

Item	Quantity	Unit Cost	Total
Production Cost			
First crop			
Seed plants (kg)	500	5.00	2,500.00
Plastic tie-tie (rolls)	1	45.00	45.00
Mangrove posts (pcs)	240	0.50	120.00
Hired labor (manday)	3	80.00	240.00
Depreciation			75.00
		Sub-total	2,980.00
Succeeding crops (2nd-9th crop)			
Plastic tie-tie (roll)	1	45.00	45.00
Hired labor/manday	3	80.00	240.00
Depreciation			75.00
		Sub-total	360.00
	Sub-total (8 crops)		2,880.00
Annual Production cost (9 crops)			5,860.00

[1]Exclude opportunity costs of labor and capital.

Table 3. Costs and returns from *G. bailinae* fixed-bottom long-line method (PhP 0.1 ha^{-1}).

Item	Quantity	Unit Cost	Total
Seedling density (kg 1000 m^{-2})	500		
Average Yield (kg-fresh) (4.5% day^{-1})*	2,220		
Less: seedling allocation (kg)	500		
Net yield (fresh, kg)	1,720		
Dry yield (20% moisture content, kg)	344	12	4,128
Net Returns			
a) First crop			
Gross returns			4,128
Less production costs			2,980
Net returns for first crop			1,148
b) Succeeding crops (2nd to 9th crop)			
Gross returns			33,024
Less production costs			2,880
Net returns for the succeeding crops			30,144
c) Annual net returns 1000 m-2			31,292
Return on investment (%)			671.50
Payback (months)			1.74

*Average SGR.

Table 4. Partial budget for a 0.1 ha "rice planting" and fixed-bottom line methods of cultivating *G. bailinae* in an estuary.

	Rice Planting*	Line cultivation
Stocking density (g m^{-2})	400	500
Yield [kg (dry)]	130	344
Revenue (9 crops at P12 kg^{-1})	14,040	37,152
Cost		
First crop		
Seed plants	800.00	1,000.00
Hired labor	0.00	240.00
Long line materials	0.00	445.00
Marketing expenses (2% of revenue)	31.20	82.56
Mangrove posts		120.00
Sub-total	831.20	1,887.56
Succeeding crops (8 crops)		
Seed plants	0.00	0.00
Hired labor	0.00	1,920.00
Long line materials	0.00	135.00
Marketing expense	249.60	660.48
Sub-total	249.60	2,715.48
Total cost	1,080.80	4,603.04
Marginal revenue	23,112.00	
Marginal cost	3,522.24	
Net benefit	19,589.76	

*Hurtado-Ponce *et al.* 1992a

Calculation of yield (dwt g m^{-2}) was based on a monthly basis to estimate the lowest and highest value. However, for purposes of computing the cost and returns, average yield (344 dwt g m^{-2}) for the 9-month culture was used. Minimum and maximum yield of 72 g mo^{-1} (6.5 dwt ha^{-1} yr^{-1}) and 660 g mo^{-1} (59.4 dwt ha^{-1} yr^{-1}) were calculated, respectively. Results of the present study are higher than those reported in *Gracilaria* by Chiang (1981) in ponds (16-43 t ha^{-1}) and Liu (1986) (3.5 t ha^{-1}) and in mudflats using floating rafts (2 t ha^{-1}) (Liu, 1987). Reports on *Gracilaria* farming in Chile using polyethylene tubes filled with sand revealed a production of 550-880 g mo^{-1} (Buschmann *et al.*, 1992). A much higher production of *Gracilaria* in tanks was reported by Hanisak 1981 and Ryther *et al.*, 1979 (127 t ha^{-1} yr^{-1}) and Ugarte and Santelices, 1992 (41 t ha^{-1} $year^{-1}$ dry). These data indicate the potential of *Gracilaria* for sustained production in a commercial cultivation.

Though an attempt to culture *Gracilaria* species on long lines was made in discharge canal of shrimp ponds in Thailand (Chandrkrachang,

1992), there were no reports on growth rate, yield and economic feasibility. Results of the study suggest that fixed-bottom long line method of cultivating *G. bailinae* in an estuary is feasible and possibly also in tidal mud flats.

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Mass	
Kilogram	kg
Gram	g
Ton (metric ton)	t
Milligram	mg
Time (same units used in both Metric and English System)	
Day	d
Hour	h
Minute	min
Second	s
Amount of substance	
Mode	mole
Temperature	
Degree celsius	°C